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(54) Title: MONOCLONAL ANTIBODIES THAT RECOGNIZE ANTIGENS ASSOCIATED WITH TUMOR METASTASIS (57) Abstract <p>This invention provides tumor metastasis-inhibiting monoclonal antibodies, mAb 41-2, mAb 50-6 and mAb 1A-5. The antigen recognized by mAb 50-6 and mAb 1A-5 has been identified as the PETA-3 antigen. The antigen recognized by mAb 41-2 has been identified as a novel tumor metastasis-associated antigen. Compositions and methods are provided that are useful for treating and diagnosing metastatic tumors.</p>		

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MONOCLONAL ANTIBODIES THAT RECOGNIZE ANTIGENS
ASSOCIATED WITH TUMOR METASTASIS

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FIELD OF THE INVENTION

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This invention relates to tumor metastasis-inhibiting monoclonal antibodies as well as antigens that are recognized by such antibodies. Compositions and methods are provided that are useful for treating and diagnosing metastatic tumors.

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DESCRIPTION OF RELATED ART

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Malignant tumors shed cells which migrate to new tissues and create secondary tumors; a benign tumor does not generate secondary tumors. The process of generating secondary tumors is called metastasis and is a complex process in which tumor cells colonize sites distant from the primary tumor. Tumor metastasis remains the major cause of deaths in cancer patients, yet the molecular mechanisms underlying tumor cell dissemination are not clearly understood. There is a need to identify new molecules that are functionally involved in cancer cell dissemination in order to gain insights into the

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basics of metastatic spread, and to identify novel prognostic indicators and/or therapeutic targets.

Metastasis is a multi-step process in which tumor cells must detach from the primary tumor, invade the cellular matrix, penetrate through blood vessels, thus enter the circulatory system (intravasate), arrest at a distant site, exit the blood stream (extravasate), and grow. See, e.g., G. L. Nicolson (1982) *Biochim. Biophys. Acta.* 695: 113-176; G. L. Nicolson and G. Poste (1983) *In. Rev. Exp. Pathol.* 25: 77-181; G. Poste and I. J. Fidler (1980) *Nature* 283: 139-145; and E. Roos (1984) *Biochim. Biophys. Acta.* 738: 263-284. Given the complexity of the process, it is likely that there are a number of genes which mediate tumor cell metastasis. Indeed, the metastatic phenotype has been correlated with expression of a variety of proteins, including proteases, adhesion molecules, and the like. However, evidence that a given protein is directly involved in dissemination is often lacking, or difficult to prove. L.A. Liotta and W. Stetler-Stevenson (1989) *J. Natl. Cancer Inst.* 81: 556-557.

The human epidermoid carcinoma, HEp-3, provides a unique model system with which to detect and characterize those genes which effect metastatic dissemination. HEp-3 cells, propagated by serial passage on the chick chorioallantoic membrane (CAM), are both tumorigenic and spontaneously metastatic (T+M+). L. Ossowski and E. Reich (1980a) *Cancer Res.* 40: 2300-2309. However, when cells are grown continuously *in vitro*, they readily form primary tumors, but progressively become non-metastatic (T+M-) with time. L. Ossowski and E. Reich (1980b) *Cancer Res.* 40: 2310-2315. With prolonged

cultivation *in vitro*, they eventually become non-tumorigenic also (T-M-). Loss of metastatic ability is reversible. T+M- cells carried on the CAM for two to three passages regain the ability to form spontaneous metastases. Thus, by altering growth conditions, the metastatic potential of these cells can be manipulated by the investigator.

Human urokinase-type plasminogen activator (uPA) was shown to be directly involved in dissemination of HEP-3, as spontaneous metastasis of HEP-3 cells in the chick embryo was inhibited by antibodies that were specific for human uPA. L. Ossowski and E. Reich (1983b) *Cell* 35: 611-619. Subsequently, it was observed that inhibition of uPA activity blocked infiltration of the CAM mesenchyme by individual HEP-3 cells. L. Ossowski (1988a) *Cell* 52: 321-328. However, active uPA appeared to be required for tumor cell intravasation but not extravasation. L. Ossowski (1988a). Thus, some other factor(s) must be also involved in HEP-3 dissemination. J.P. Quigley et al. (1988) *Ciba Foundation Symposium* 141: 22-47 and Brooks et al. (1993) *J. Cell Biol.* 122 (6): 1351-1359 describe the generation of monoclonal antibodies (mAbs), using "subtractive immunization", which recognize cell surface antigens expressed on HEP-3 cells and inhibit tumor metastasis in the CAM model.

Angiogenesis is the growth of new blood vessels from pre-existing vessels. Endothelial cells which line the vessel walls degrade the surrounding basement membrane and migrate into the surrounding connective tissue. The migrating endothelial cells form cords, which eventually develop lumina and become patent vessels. Angiogenesis plays a vital role in normal

physiological processes (e.g., wound healing, ovulation). Inappropriate angiogenesis is involved in a number of pathological processes, such as cancer metastasis, diabetic retinopathy, etc.

5 The present invention has generated monoclonal antibodies by subtractive immunization, which inhibit tumor metastasis and/or angiogenesis. The antigens that are recognized by such monoclonal antibodies have been identified as well.

10 SUMMARY OF THE INVENTION

One embodiment of the present invention provides tumor metastasis-inhibiting monoclonal antibodies 41-2 and 50-6. Functional derivatives and fragments of such monoclonal antibodies are also contemplated by the present invention.

15 Another embodiment of the present invention provides hybridoma cell lines 41-2 (ATCC #) and 50-6 (ATCC #), which produce mAb 41-2 and mAb 50-6, respectively.

20 Another embodiment of the present invention provides the antigens recognized by the monoclonal antibodies of the present invention, more particularly, the target antigen of mAb 41-2.

25 Another embodiment of the present invention is directed to antibodies raised against the target antigen of mAb 41-2, as well as antibodies raised against the target antigen of mAb 50-6.

30 Another embodiment of the present invention is directed to nucleic acid molecules coding for the target antigen of mAb 41-2 or a portion thereof. Expression

vector and host cells are also contemplated by the present invention.

In a further aspect of the present invention, pharmaceutical compositions are provided.

5 A pharmaceutical composition of the present invention can include the PETA-3 antigen or a portion thereof, or the antigen recognized by mAb 41-2 or a portion thereof.

10 A pharmaceutical composition of the present invention can also include an antibody directed against PETA-3 or the target antigen of mAb 41-2.

15 A preferred pharmaceutical composition of the present invention includes a tumor-metastasis-inhibiting antibody. Preferred tumor-metastasis-inhibiting antibodies include mAb 41-2, mAb 50-6 and mAb 1A-5.

20 A further aspect of the invention provides methods of diagnosing metastatic tumors in a subject by detecting the expression of at least one of the metastasis-associated antigens identified by the present invention, including the PETA-3 antigen or the antigen recognized by mAb 41-2. The detection can be accomplished by using an antibody or a nucleic acid probe.

25 Another embodiment of the present invention provides methods of treating a metastatic tumor in a subject by administering to the subject a therapeutically effective amount of a tumor metastasis-inhibiting antibody of the present invention.

30 Another embodiment of the present invention provides methods of treating a metastatic tumor in a subject by administering to the subject a therapeutically

effective amount of an antisense molecule of the PETA-3 gene or the gene encoding the target antigen of mAb 41-2.

In still another embodiment, the present invention is directed to methods of treating aberrant PETA-3-dependent angiogenesis in a subject by administering a therapeutically effective amount of an angiogenesis-inhibiting antibody directed against PETA-3 or a functional derivative of such antibody.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1A depicts the nucleotide sequence (**SEQ ID NO: 1**) of a cDNA clone isolated in expression cloning using mAb 50-6;

Figure 1B depicts the encoded amino acid sequence (**SEQ ID NO: 2**) of the cDNA clone isolated in expression cloning using mAb 50-6.

Figure 2 depicts the immunocytochemical localization of PETA-3 on HEP-3 cells and COS transfectants. Cells were plated onto poly-lysine-coated glass cover slips, fixed 24 hours later, and then immunostained with mAb 50-6 or normal mouse IgG. Signals were detected with horseradish peroxidase-conjugated goat anti-mouse IgG and chloronaphthol. All images were photographed with transmitted light. (a) HEP-3 cells incubated with mAb 50-6; (b) HEP-3 cells incubated with normal mouse IgG; (c) COS cells transiently transfected with PETA-3 cDNA and incubated with mAb 50-6; (d) COS cells transiently transfected with PETA-3 cDNA and incubated with normal mouse IgG; (e) COS cells transiently transfected with vector (pcDNA I) alone and incubated with mAb 50-6. A 20 μ m bar is shown in the lower right of each panel.

Figure 3 depicts the recognition of PETA-3/CD151 by mAb 50-6 and mAb 1A5. COS-7 cells were transiently transfected with the cloned PETA-3/CD151 cDNA, or vector (pcDNA I) alone. Lysates of HEP-3 cells and the transfected COS cells were resolved on SDS-PAGE, and analyzed by Western blotting. Panel A: Western blot of HEP-3 cell lysate (10 µg; lane 1) lysates from control (COS/pcDNA I; 20 µg; lane 2) and PETA-3-transfectants (COS/PETA-3; 20 µg; lane 3) probed with mAb 50-6. Panel B: Western blot of HEP-3 cell lysate (0.5 µg; lane 1) and lysates from control (COS/pcDNA I; lane 2; 2.5 µg) and PETA-3 transfectants (COS/PETA-3; lane 3; 2.5 µg) probed with mAb 1A5. In both panels the signals were visualized with a peroxidase-conjugated goat anti-mouse IgG by chemiluminescence. PETA-3 is identified with an arrow at 29 kD. Molecular weight markers, in kilodaltons (kD) are shown to the right.

Figure 4 depicts the relative immunostaining of human breast carcinoma cell lines with mAb 41-2.

Figure 5 depicts the relative expression of the antigen recognized by mAb 41-2 in whole-cell ELISA of HEP-3, MDA-MB-231, and normal human mammary epithelial cells (HMEC).

Figure 6 depicts the western blot analysis of HEP-3 and MDA-MB-231 lysates with mAb 41-2.

Figure 7 depicts whole cell ELISA of cell-surface PETA-3 expression by equal numbers (2×10^4) of HEP-3 cells, MDA-MB-231 human breast carcinoma cells, and normal human mammary epithelial cells (HMEC; Clonetics). Background signals from normal mouse IgG controls have been subtracted from the data shown.

Figure 8 graphically depicts a Matrigel invasion assay.

Figure 9 depicts the inhibition of Matrigel invasion by mAb 41-2. MDA-MB-231 cells and HEp-3 cells were placed in Matrigel invasion chambers (Collaborative Research) in the presence of mAb 41-2 or normal mouse IgG. Data are expressed as the number of cells on the underside of the filter (i.e., invasive cells) per microscopic field.

Figure 10 graphically depicts a cell migration assay.

Figure 11 depicts the inhibition of HEp-3 migration by mAb 50-6 and mAb 1A5. Experiments were conducted in triplicate.

Figure 12A-12C depicts the effects of PETA-3/CD151 expression on HeLa cell migration. HeLa cells were co-transfected with the cloned PETA-3/CD151 cDNA and pSV2neo. Controls were co-transfected with pcDNA I and pSV2neo. Two PETA-3 over-expressing clones (PB6_{HI} and PC3_{HI}) and two control clones (NB11_{LO} and NB17_{LO}) were selected.

Figure 12A depicts the amount of cell surface PETA-3 expressed by the HeLa transfectants and HEp-3 cells, determined by whole-cell ELISA on fixed, non-permabilized cells and expressed as the optical density (OD) of the chromogen at 405 nm.

Figure 12B depicts the *in vitro* growth rates of the HeLa transfectants, determined by plating 2×10^4 cells into each of two wells in a 24-well plate. At the times indicated, the cells were trypsinized and counted. Results are reported as number of cells per well.

Figure 12C depicts the inhibition of HeLa migration by mAb 50-6 and mAb 1A5. Experiments were conducted in triplicate.

Figure 13 depicts whole-cell ELISA of expression of the $\alpha_3\beta_1$ integrin heterodimer by equal numbers (2×10^4) of MDA-MB-231 human breast carcinoma cells, HT1080 human fibrosarcoma cells, HEP-3 cells, and the PETA-3 overexpressing HeLa clones (PB6_{HI}, PC3_{HI}) and control HeLa clones (NB11_{LO}, NB17_{LO}). MAb 1992 was used to specifically identify the heterodimer. Data represent the amount of chromogen released in the assay and are expressed as the optical density (OD) at 405 nm.

Figure 14 depicts PETA-3 expressed by HEP-3 cells associates with the $\alpha_3\beta_1$ integrin heterodimer. HEP-3 cell lysates were incubated with mAb 1A5 (lane 2), mAb 1992 (specific for the $\alpha_3\beta_1$ heterodimer; lane 3), or normal mouse IgG (NM; lane 4). Immune complexes were collected on GammaBind Plus Sepharose, resolved by SDS-PAGE and transferred to nitrocellulose. The blot was incubated with unlabeled mAb 1A5 as a primary antibody, followed by horseradish peroxidase-conjugated goat anti-mouse IgG. Signals were visualized by chemiluminescence. As a control, a NM immunoprecipitate was probed with NM IgG and peroxidase-conjugated goat anti-mouse IgG (lane 5). A sample of the original HEP-3 lysate is shown in lane 1. The PETA-3 signal is indicated by *. Molecular weight markers in kilodaltons (kD) are shown to the left.

Figure 15A depicts the effects of mAb 50-6 and mAb 1A5 on cell adhesion to fibronectin of HEP-3 cells, PETA-3-overexpressing HeLa clones (PB6_{HI}, PC3_{HI}) and control HeLa clones (NB11_{LO}, NB17_{LO}).

Figure 15B depicts the effects of mAb 50-6 and mAb 1A5 on cell adhesion to vitronectin of HEp-3 cells, PETA-3-overexpressing HeLa clones (PB6_{HT}, PC3_{HT}) and control HeLa clones (NB11_{Lo}, NB17_{Lo}).

5 **Figure 15C** depicts the effects of mAb 41-2 on cell adhesion of HEp-3 cells to fibronectin

Figure 16 graphically depicts the *in vivo* angiogenesis assay.

10 **Figure 17** depicts the effect of bFGF on neovascularization of type I collagen matrix. Rafts are examined *in situ* after 66 hours incubation on the CAM using a dissecting microscope. Each box in the top mesh is scored as positive or negative based on the presence or absence of vessels that have penetrated up to the
15 plane of the top mesh. (A) No addition and (B) bFGF (16 µg/ml). Arrowheads indicate representative nascent blood vessels in the plane of the top mesh. Selected vessels only are labeled.

20 **Figure 18** depicts the effects of mAb 50-6 on basic fibroblast factor (bFGF)-induced angiogenesis on the chicken chorioallantoic membrane (CAM).

25 **Figure 19** depicts the Western Blot analysis of lysates of HEp-3 cells and normal mouse lung probed with mAb 50-6 and mAb1A-5. PETA-3 is seen at 29 kD in the HEp-3 lysates.

30 **Figure 20** depicts the alignment of the amino acid sequences of human and mouse PETA-3/CD151. Amino acids are indicated by the standard IUPAC one-letter code. Putative transmembrane domains are underlined. The extracellular loops are double underlined. Substitutions are indicated with a vertical line. Non-

conservative amino acid substitutions in the human sequence are shaded in gray.

Figure 21 depicts the alignment of the amino acid sequences of the large extracellular loops of human PETA-3, CD9 and CD63. Amino acids are indicated by the standard IUPAC one-letter code. Amino acids shaded in gray are the non-conservative substitutions identified in the human sequence when compared to the mouse PETA-3 homologue.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to isolated monoclonal antibodies that inhibit tumor metastasis. The present invention further relates to tumor metastasis-associated antigens recognized by such monoclonal antibodies, the nucleic acid molecules encoding such antigens, as well as other antibodies raised against such antigens. The present invention also provides pharmaceutical compositions and methods useful for diagnosing and treating metastatic cancers.

One embodiment of the present invention is directed to monoclonal antibodies 41-2 and 50-6.

According to the present invention, mAb 41-2, and mAb 50-6 inhibit metastasis of tumors, including tumors of a tissue origin of the epiderm, breast, lung, liver, kidney, epithelial, thyroid, leukemic, pancreatic, endometrial, ovarian, cervical, skin, colon or lymphoid tissues. The present invention has also demonstrated that mAb 50-6 inhibits growth factor-induced angiogenesis.

The metastasis inhibitory effects of these monoclonal antibodies can be attributed to binding of the

mAbs to specific tumor metastasis-associated antigens and interfering with the function of such antigens in tumor metastasis.

5 The term "tumor metastasis-associated antigen" as used herein, includes antigens that are specifically expressed in metastatic tumor cells and essentially not expressed in non-metastatic tumor cells or normal cells; antigens that are overexpressed in metastatic tumor cells relative to non-metastatic tumor cells or normal cells; 10 and antigens that are expressed similarly in metastatic tumor cells and non-metastatic tumor cells, but are required for the process of tumor metastasis.

 As demonstrated by the present invention, both the antigen recognized by mAb 41-2 and the antigen 15 recognized by mAb 50-6 are expressed at a substantially higher level on metastatic HEP-3 cells than on non-metastatic HEP-3 cells. The antigen recognized by mAb 41-2 is also expressed on metastatic human breast carcinoma cells at a substantially higher level than on 20 less metastatic human breast carcinoma cells or normal human mammary epithelial cells. In addition, the antigen recognized by mAb 50-6 is expressed at a substantially higher level on human mammary metastatic carcinoma cells than on normal human mammary cells.

25 Functional derivatives of the instant monoclonal antibodies are also contemplated by the present invention.

 "Functional derivatives" refer to antibody 30 molecules or fragments thereof that are derived from the instant monoclonal antibodies and that have retained the antigen specificity of the instant monoclonal antibodies. Examples of functional derivatives include Fab, Fab',

F(ab')₂ of the present mAbs, single chain antibodies, humanized antibodies and the like.

5 A single-chain antibody (SAb) is created by fusing together the variable domains of the heavy and light chains using a short peptide linker, thereby reconstituting an antigen binding site on a single molecule. Such single-chain antibody variable fragments (Fvs) can be fused to all or a portion of the constant domains of the heavy chain of an immunoglobulin molecule, 10 if necessary. The use of sAb avoids the technical difficulties in the introduction of more than one gene construct into host cells. Single chain antibodies and methods for their production are known in the art. See, e.g., Bedzyk et al. (1990) *J. Biol. Chem.*, 265:18615; 15 Chaudhary et al. (1990) *Proc. Natl. Acad. Sci.*, 87:9491; U.S. Patent No. 4,946,778 to Ladner et al.; and U.S. Patent No. 5,359,046 to Capon et al.

20 The murine monoclonal antibodies of the present invention can be humanized to reduce the immunogenicity for use in humans. One approach is to make mouse-human chimeric antibodies having the original variable region of the murine mAb, joined to constant regions of a human immunoglobulin. Chimeric antibodies and methods for their production are known in the art. See, e.g., 25 Cabilly et al., European Patent Application 125023 (published Nov. 14, 1984); Taniguchi et al., European patent Application 171496 (published Feb. 19, 1985); Morrison et al., European Patent Application 173494 (published Mar. 5, 1986); Neuberger et al., PCT 30 Application WO 86/01533, (published Mar. 13, 1986); Kudo et al., European Patent Application 184187 (published Jun. 11, 1986); Robinson et al., International Patent

Publication #PCT/US86/02269 (published 7 May 1987); Liu et al., *Proc. Natl. Acad. Sci. USA* 84:3439-3443 (1987); Sun et al., *Proc. Natl. Acad. Sci. USA* 84:214-218 (1987); Better et al., *Science* 240:1041-1043 (1988). These
5 references are incorporated herein by reference.

Generally, DNA segments encoding the H and L chain antigen-binding regions of the murine mAb can be cloned from the mAb-producing hybridoma cells, which can then be joined to DNA segments encoding C_H and C_L regions of a
10 human immunoglobulin, respectively, to produce murine-human chimeric immunoglobulin-encoding genes.

Humanized antibodies can be made using a second approach, i.e., to construct a reshaped human antibody, which has been described in, e.g, Maeda et al., *Hum. Antibod. Hybridomas* 2: 124-134, 1991, and Padlan, *Mol. Immunol.* 28: 489-498, 1991.
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The monoclonal antibodies of the present invention can be prepared from the culture supernatant of hybridoma cell lines 41-2 (deposited with American Type Culture Collection on June 15, 1999, ATCC #) and 50-6
20 (deposited with American Type Culture Collection on June 15, 1999, ATCC #), respectively. Such hybridoma cell lines form another embodiment of the present invention.

The hybridoma cell line of the present invention have been generated by the "subtractive immunization" procedure. Briefly, recipient mice were first inoculated with non-metastatic human epidermoid carcinoma HEP-3 cells, followed by injections of an immunosuppressive (tolerizing) agent cyclophosphamide.
25 The tolerizing agent suppressed the immune response in the recipient mice towards dominant immundeterminants present on the non-metastatic tumor cells. The tolerized
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mice were then immunized with metastatic HEP-3 cells to generate an immune response to antigenic determinants present on the metastatic tumor cells. A boosting injection with metastatic tumor cells was made afterwards. Subsequently, monoclonal antibody-producing spleen cells from the immunized mice were isolated and fused with the immortalized murine myeloma cell line NSO to produce hybridomas. The procedure for making hybridomas is well known in the art and can be found in, e.g., Harlow, E. and Lane, D., *Antibodies: A Laboratory Manual*, Cold Spring Harbor Press, 1988. The supernatants of the cultured hybridomas were tested in a whole-cell ELISA assay for differential reactivity toward metastatic tumor cells over non-metastatic tumor cells, which cells were used in the subtractive immunization procedure. In the whole-cell ELISA assay, tumor cells were fixed but not permeablized, such that only cell surface antigens were accessible for binding by antibodies in the hybridoma supernatants. Hybridomas producing antibodies which recognized antigenic determinants that were expressed on metastatic tumor cells at a substantially higher level than on non-metastatic cells were identified. Clones 50-6 and 41-2 were obtained after limiting dilution.

The hybridomas of the present invention can be grown in various tissue culture flasks, yielding supernatants with variable concentrations of antibodies. Hybridoma cells can be injected into animals to produce inflammatory ascites. Antibody-containing ascites can be harvested 8-12 days after intraperitoneal injection. The ascites contain a higher concentration of antibodies, but

include both monoclonals and immunoglobulins from the animal.

Monoclonal antibodies in the hybridoma supernatant or in the ascites can be purified by a variety of conventional protein purification methods, for example, affinity chromatography. Alternatively, hybridoma supernatant or the ascites can be used directly in place of the monoclonal antibodies under certain circumstances. Those skilled in the art can determine whether purification of the monoclonal antibodies is required for a particular use.

Another embodiment of the present invention is directed to the antigens recognized by the monoclonal antibodies of the present invention, i.e., the target antigens.

More particularly, the present invention has identified and isolated the target antigen of mAb 50-6. Such antigen has been determined to be identical to a protein known as PETA-3/CD151. PETA-3/CD151 has not been associated with tumor metastasis or angiogenesis prior to the present invention.

Furthermore, the present invention provides the isolated target antigen of mAb 41-2.

According to the present invention, the target antigen of mAb 41-2 is associated with tumor metastasis. Such antigen has been found to be expressed on the surface of metastatic tumor cells, e.g., metastatic mammary carcinoma, at a substantially higher level than on the surface of non-metastatic tumor cells or normal cells. By "substantially higher" it means at least about 20% higher, preferably, at least about 25% higher, more preferably, at least about 35% higher.

Such antigen has been substantially purified from metastatic Hep-3 cell lysate using mAb 41-2 (ATCC#). The antigen appears as a single band on a SDS-PAGE under both reducing and non-reducing conditions with an
5 approximate Mw of about 180 kD. The immun-purified antigen was subjected to Edman degradation, and the subsequent sequencing analysis revealed a N-terminal sequence of FEIALPRESQITVLQ(I)KXGT (SEQ ID NO: 3) (residue #15 is either Q or I; residue 17 has not been
10 determined). Comparison of this partial amino acid sequence with known sequences in the NCBI database has revealed that this antigen has not been previously identified.

Another embodiment of the present invention is
15 directed to antibodies raised against the target antigen of mAb 41-2 and antibodies raised against the target antigen of mAb 50-6. According to the present invention, such antibodies do not include mAb 1A-5 (produced by hybridoma 1A-5 (ATCC#) and described by Brooks et al.,
20 JCB 122:1351, 1993), 11B1.G4 or 14A2.H1 (the latter two described by Fitter et al., Blood 4: 1348-1355, 1995). However, the present invention contemplates pharmaceutical compositions which include mAb 1A-5 and methods for diagnosing or treating metastatic tumors by
25 using mAb 1A-5.

The antigen, preferably substantially purified, can be injected, alone or with adjuvant, into an animal to raise antibodies. Polyclonal antibodies can be purified from the serum of the immunized animal.
30 Monoclonal antibodies can be prepared from hybridomas which are produced by fusing the antibody-producing lymphocytes from the immunized animal and an immortal

cell line. Procedures for raising and preparing antibodies are well known in the art and can be found in, e.g., Harlow, E. and Lane, D., *Antibodies: A Laboratory Manual*, Cold Spring Harbor Press, 1988.

5 Functional derivatives and fragments of antibodies raised against the target antigen of mAb 41-2 are also contemplated by the present invention.

10 Another embodiment of the present invention is directed to nucleic acid molecules coding for the target antigen of mAb 41-2 or a portion thereof.

 "A portion" of an antigen as used herein, refers to a peptide fragment of a sufficient length to be antigenic. Generally speaking, the peptide fragment consists of at least 7 or 8 amino acids.

15 Nucleic acid molecules coding for the target antigen of mAb 41-2 can be obtained by a variety of well known methods. For example, degenerate oligonucleotides can be synthesized based on the partial amino acid sequence of the antigen, FEIALPRESQITVLQ(I)KXGT (**SEQ ID**
20 **NO: 3**). Such oligonucleotides can be used to screen a cDNA library made from metastatic tumor cells. Alternatively, mAb 41-2 (ATCC#) can be used to screen the encoding gene by, e.g., expression cloning. Expression cloning is well known in the art and is also
25 described in Example 2 relating to the isolation of the target antigen of mAb 50-6.

30 In another embodiment, nucleotide sequences encoding the target antigen of mAb 41-2 or a portion thereof, have been inserted into an expression vector for production of the antigen or the portion thereof.

 According to the present invention, a desired nucleotide sequence can be inserted into a selected

vector in an operable linkage to a promoter. For expression in a eukaryotic cell, viral vectors are preferred, e.g., a retroviral, adenoviral, or herpes simplex viral vectors. Yeast vectors are also contemplated. For expression in a prokaryotic cell, phage vectors are preferred, such as a λ phage vector. Baculovirus-based vectors are also useful for recombinant production of large amounts of a protein or portions thereof (O'Reilly et al., *Baculovirus Expression Vectors: A laboratory Manual*, pp. 107-122, CRC Press, Boca Raton, FL, 1992).

The present invention also contemplates host cells transformed with the above-described expression vector. The host cell lines which can be employed include, but are not limited to, eukaryotic cells, e.g., COS cells such as COS-7, CHO cells such as CHO-1, NIH 3T3 cells, yeast cells such as strains of *Saccharomyces* and *Pichia pastoris*, insect cells such as *Spodoptera frugiperda*; and prokaryotic cells, e.g., strains of *E. coli*, strains of *Pseudomonas* such as *Pseudomonas aeruginosa* or strains of *Bacillus*.

Recombinant expression allows production and purification of large amounts of the target antigen or a portion thereof, which can be used to raise antibodies, which, in turn, can be used for diagnosing and treating metastatic tumors. In addition, recombinantly produced proteins in large quantity permits large scale screening of therapeutic drugs for treating metastatic tumors.

A further aspect of the invention is directed to pharmaceutical compositions.

A pharmaceutical composition of the present invention can include PETA-3 or a portion thereof, the

antigen recognized by mAb 41-2 or a portion thereof, or combinations thereof. Such pharmaceutical compositions can be used as immunogens to raise antibodies in a laboratory animal, or as vaccines to provoke immune
5 responses in a recipient subject which facilitate the elimination of the metastatic tumor cells from the recipient subject.

A pharmaceutical composition of the present invention can also include an antibody directed against
10 the PETA-3 antigen or the target antigen of mAb 41-2, functional derivatives of such antibodies, or combinations thereof.

Preferred antibodies to be used in pharmaceutical compositions include those antibodies that
15 inhibit tumor metastasis, e.g., mAb 41-2, mAb 50-6 and mAb 1A-5. Inhibition of tumor metastasis can be determined by a number of assays, such as the migration assay, the invasion assay or the chick CAM assay as described in the Examples hereinafter.

The pharmaceutical compositions of the present invention can include other substances such as cytokines, adjuvants and pharmaceutically acceptable carriers. As
20 used herein, a pharmaceutically acceptable carrier includes any and all solvents, including water, dispersion media, culture from cell media, isotonic
25 agents and the like that are non-toxic to the host. Preferably, it is an aqueous isotonic buffered solution with a pH of around 7.0. The use of such media and agents in therapeutic compositions is well known in the
30 art.

A further aspect of the invention is directed to methods of diagnosing metastatic tumors in a subject by detecting the expression of at least one of the metastasis-associated antigens identified by the present invention, i.e., the PETA-3 antigen or the antigen
5 recognized by mAb 41-2.

"Metastatic tumors" as used in the present invention include both tumors at the primary site capable of metastasizing and metastasized tumors at a secondary
10 site. Such metastatic tumors can be of a tissue origin of the lung, liver, kidney, mammary gland, epithelial, thyroid, leukemic, pancreatic, endometrial, ovarian, cervical, skin, colon and lymphoid tissues.

A "subject" as used herein refers to a
15 mammalian subject, including humans, dogs, monkeys, cows and the like, with the exception of mice.

The expression of a tumor metastasis-associated antigen can be detected by using an antibody directed towards such antigen. Both polyclonal antibodies and
20 monoclonal antibodies can be employed. Preferred antibodies for use in diagnosis include mAb 41-2 (ATCC#), mAb 50-6 (ATCC#) and mAb 1A-5.

A sample is taken from the subject, e.g., a biopsy specimen taken from tissue suspected of having a
25 metastatic tumor. Generally, the sample is treated before an assay is performed. Assays which can be employed include ELISA, RIA, EIA, Western Blot analysis, immunohistological staining and the like. Depending upon the assay used, the antigens or the antibodies can be
30 labeled by an enzyme, a fluorophore or a radioisotope. See, e.g., Coligan et al. *Current Protocols in*

Immunology, John Wiley & Sons Inc., New York, New York (1994); and Frye et al., *Oncogen* 4: 1153-1157, 1987.

The treatment of the sample may vary depending on the assay that is used to detect an antigen. For example, cells of tissue biopsy can be lysed and the cell lysates are used in e.g., Western Blot analysis. For assays such as the Whole Cell ELISA assay, cells can be washed with, e.g., PBS, and then fixed with 0.25% glutaraldehyde in PBS before the assay.

The expression of a tumor metastasis-associated antigen can also be detected by using a nucleic acid probe, e.g., probes that hybridize to the mRNA of a tumor-metastasis antigen, PETA-3 or the target antigen of mAb 41-2.

A PETA-3 nucleic acid probe can be made from any region of PETA-3 RNA or DNA. A probe for detecting the mRNA of the target antigen of mAb 41-2, can be made based on the partial amino acid sequence of such antigen, i.e., SEQ ID NO: 3. In order to produce a detectable signal, the probe is generally at least about 14 nucleotides, and labeled with either an enzyme (such as horse radish peroxidase, alkaline phosphatase, glucose oxidase and β -galactosidase), a fluorophore or a radioisotope.

Detection of the expression of an antigen with a nucleic acid probe can be achieved by a variety of hybridization procedures which are well known in the art. For example, mRNA can be extracted from tissue specimen and analyzed in, e.g., Northern Blot analysis. Alternatively, in situ hybridization procedure can be employed, in which lysis of cells and isolation of RNA are not necessary. See, e.g., *Current Protocols in*

Molecular Cloning (Ausubel et al., John Wiley & Sons, New York).

5 The expression of an antigen detected by using any of the above-described methods, is compared with the expression of the same antigen in the normal part of the tissue. A substantial increase in the level of expression of at least one of the PETA-3 antigen or the target antigen of mAb 41-2, when compared with the expression in the normal tissue, is indicative of a
10 metastatic tumor. By "a substantial increase" it means an increase of at least about 20%, preferably, at least about 25%, more preferably, at least about 35%.

 A further aspect of the invention is directed to methods of treating metastatic tumors.

15 By "treating a metastatic tumor", it means that the metastasis of the tumor is prevented, delayed, or inhibited.

 "Metastatic tumors" as used in the present invention include both tumors at the primary site capable
20 of metastasizing and metastasized tumors at a secondary site. Such metastatic tumors can be of a tissue origin of the lung, liver, kidney, mammary gland, epithelial, thyroid, leukemic, pancreatic, endometrial, ovarian, cervical, skin, colon and lymphoid tissue.

25 A "subject" which can be treated can be any mammalian subject, including humans, dogs, monkeys, cows and the like, with the exception of mice.

 One embodiment of the present invention provides methods of treating a metastatic tumor in a
30 subject by administering to the subject a therapeutically effective amount of a tumor metastasis-inhibiting antibody of the present invention.

Tumor metastasis-inhibiting antibodies of the present invention have been described hereinabove. Preferred tumor metastasis-inhibiting antibodies include mAbs 41-2, 50-6 and 1A-5, as well as functional
5 derivatives of such mAbs.

Another embodiment of the present invention provides methods of treating a metastatic tumor in a subject by administering to the subject a therapeutically effective amount of an antisense molecule of the PETA-3
10 gene or the gene encoding the target antigen of mAb 41-2.

An antisense DNA can have at least about 10 nucleotides, preferably, at least about 15 or 17 nucleotides, more preferably, at least about 50 nucleotides. The antisense DNA is preferably inserted
15 into an expression vector in an operable linkage to a promoter which can effect the transcription of the antisense RNA. Any of the well-known gene therapy vectors, such as retroviral, adenoviral, adeno-associated viral, herpes simplex viral vectors, can be used for
20 practicing the methods of the present invention.

A tumor metastasis-inhibiting antibody or an antisense molecule can be administered alone or together with a pharmaceutically acceptable carrier. A pharmaceutically acceptable carrier includes all
25 solvents, such as fats, oils, water, saline solutions, lipids, liposomes, resins, binders, fillers, dispersion media, cell culture media, and the like, or combinations thereof, that are non-toxic to the recipient subject.

In accordance with the present invention, the
30 active ingredients can be combined with the carrier in any convenient and practical manner, e.g., by solution,

suspension, emulsification, admixture, encapsulation, absorption and the like, and if necessary, by shaping the combined compositions into pellets or tablets. Such procedures are routine for those skilled in the art.

5 Dosages of a compound (i.e., an antibody or an antisense molecule) to be therapeutically effective depend on the disease state and other clinical factors, such as weight and condition of the subject, the subject's response to the therapy, the type of
10 formulations and the route of administration. The precise dosage of a compound to be therapeutically effective can be determined by those skilled in the art. As a general rule, the therapeutically effective dosage of a compound can be in the range of about 0.5 μ g to about
15 2 grams per unit dosage form. A unit dosage form refers to physically discrete units suited as unitary dosages for mammalian treatment: each unit containing a pre-determined quantity of the active material calculated to produce the desired therapeutic effect in association
20 with any required pharmaceutical carrier. The methods of the present invention contemplate single as well as multiple administrations, given either simultaneously or over an extended period of time.

25 The administration of a tumor metastasis-inhibiting antibody or an antisense molecule may be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. Preferably, the antibodies of the present invention are administered to a
30 patient by subcutaneous (s.c.), intraperitoneal (i.p.), intra-arterial (i.a.), or intravenous (i.v.) injection.

In still another embodiment, the present invention is directed to methods of treating an aberrant PETA-3-dependent angiogenesis in a subject by administering a therapeutically effective amount of an angiogenesis-inhibiting antibody directed against PETA-3 or a functional derivative of such antibody.

The present invention has demonstrated that mAb 50-6 inhibits PETA-3-dependent growth-factor induced angiogenesis, in particular, bFGF-induced angiogenesis. By the same assay, one skilled in the art can readily determine whether an antibody directed against PETA-3 can inhibit angiogenesis. Such angiogenesis-inhibiting antibodies, including mAb 50-6, as well as the functional derivatives thereof, can be used in the methods of treating an aberrant PETA-3-dependent angiogenesis in a subject.

According to the present invention, "an aberrant PETA-3-dependent angiogenesis" include abnormal growth of blood vessels as occurring in patients with hemangiomas, arthritis, ocular neovascularization or the like, which is dependent on the function of PETA-3. According to the present invention, one example of an aberrant PETA-3-dependent angiogenesis is aberrant growth factor-induced angiogenesis, which may be induced by growth factors such as fibroblast growth factor, angiogenin, transforming growth factor α , transforming growth factor β , GCSF, vascular endothelial growth factor, among others.

By "treating an aberrant PETA-3-dependent angiogenesis", is meant that the function of PETA-3 is inhibited by the antibody and the aberrant angiogenesis is prevented or inhibited.

An angiogenesis-inhibiting antibody can be administered as any other antibody described herein. The amount of antibody to be administered that is therapeutically effective depends on the disease state and other clinical factors, such as weight and condition of the subject, the subject's response to the therapy, the type of formulations and the route of administration. The precise dosage of an antibody to be therapeutically effective can be determined by those skilled in the art. As a general rule, the therapeutically effective dosage of a compound can be in the range of about 0.5 μ g to about 2 grams per unit dosage form. A unit dosage form refers to physically discrete units suited as unitary dosages for mammalian treatment: each unit containing a pre-determined quantity of the active material calculated to produce the desired therapeutic effect in association with any required pharmaceutical carrier. The methods of the present invention contemplate single as well as multiple administrations, given either simultaneously or over an extended period of time.

All the publications mentioned in the present disclosure are incorporated herein by reference. The terms and expressions which have been employed in the present disclosure are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

The present invention is further illustrated by the following examples.

EXAMPLE 1
MATERIALS AND METHODS

Materials

Human breast adenocarcinoma cells MDA-MB 231, MDA-MB 468 and MDA-MB 361 were obtained from American Type Culture Collection (Rockville, MD). HEP-3 cells were maintained as monolayer cultures and were obtained from solid HEP-3 tumors that were serially passaged on the chorioallantoic membranes (CAMs) of chick embryos as described by Ossowski et al., *Cancer Res.* 40: 2300-2309 (1980) and Ossowski et al., *Cancer Res.* 40: 2310-2315 (1980). The metastatic variant of HEP-3 cells, M⁺ HEP-3, was cultured for 25 d or less before use. The nonmetastatic variant, M⁻ HEP-3, was maintained in culture for at least 60-80 d before use. Cells were maintained in DMEM (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FBS (Hyclone, Logan, UT), sodium pyruvate, and pen/strep (Sigma Chem. Co., St. Louis, MO). All cells were grown in a humidified 5% CO₂ atmosphere at 37°C in 100-mm tissue culture plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ).

Fertilized COFAL-negative eggs were obtained from SPAFAS (Norwich, CT) and incubated at 37°C with 60% humidity until use for chick embryo metastasis assays.

Whole Cell ELISA

Subconfluent monolayer cultures of HEP-3 cells, as well as various other cell types, were washed 3 x in sterile PBS, removed with nonenzymatic cells dissociation solution, washed in sterile PBS and resuspended in DMEM

containing 10% FBS. Cells were added ($2 \times 10^4/0.1$ ml/well) to 96 well tissue culture plates and allowed to attach for 24-36 h in a humidified atmosphere of 5% CO₂ at 37°C. Cells were washed 3 X with PBS and fixed with 0.25% glutaraldehyde in PBS (0.1 ml/well) for 10 min. The plates were washed 3 X with PBS, blocked with 1.0 M glycine, pH 8.1 (0.1 ml/well) for 2 h at room temperature, washed 3 X with PBS and used directly or stored in 0.1% sodium azide in PBS at 4°C. Stored plates were washed 3 X with PBS (no azide) just before assay.

The washed, cell-coated wells were blocked with DMEM containing 10% FBS (0.2 ml/well) overnight at 4°C. The primary antibody (hybridoma supernatants or purified mAbs) or the control antibody (normal mouse IgG) was added to the wells (50 µl/well) at a concentration of 1 µg/ml and incubated for 2 h at room temperature. ELISA plates were washed 3 times with PBS containing 0.5% BSA, and then incubated with a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (dilution 1:100 in 1% BSA/PBS) (Sigma Chemical Co., St. Louis, MO) for 1 h at room temperature. The plates were washed and 100 µl of the substrate, o-phenylene diamine was added to each well. After incubation for 30 min at 37°C, the ELISA plates were read at 405 nm on a Titer Tek Multiscan plate reader, and the amount of chromophore generated from the substrate was quantitated. Nonspecific binding was determined using preimmune sera or normal mouse IgG (Cappel, Organon Teknika, Durham, NC) and subtracted from the experimental samples. ELISA ratios were calculated as follows: $(OD_{405} \text{ M}^* \text{HEp-3} / OD_{405} \text{ M}^* \text{HEp-3})$.

Western Blot Analysis

Cells were grown to confluence in 100-mm tissue culture plates and washed 3 X with PBS. Whole cell lysates were prepared by the addition of 1 ml of lysis buffer (0.1 M Tris pH 8.0 and 1.0% Triton-100 supplemented with a protease inhibitor mixture including 5 mM EDTA, 20 μ g/ml Leupeptin, 30 μ g/ml TPCK, 20 μ g/ml SBTI and 20 U/ml Aprotinin). The lysates were incubated with agitation for 10 min at 4°C and centrifuged at 3,000 g for 10 min to remove detergent insoluble materials. Lysates having about 20 μ g proteins were electrophoresed through either a 10% or 7.5% SDS-PAGE gel under nonreducing conditions. The proteins were transferred onto nitrocellulose membranes. The blots were blocked with 5% non-fat milk, incubated with either mAbs or normal mouse IgG at 4°C overnight, and washed 4 X with 0.1% Tween-20 in PBS for 15 min. Peroxidase-conjugated goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO) was added as secondary antibody. The immunoblots were washed and then developed with the chemiluminescence ECL system (Amersham International, UK) per manufacturer's protocol.

EXAMPLE 2
Isolation of Monoclonal
Antibodies 41-2, 50-6 and 1A5

5 MAB 41-2, mAb 50-6 and mAb 1A5 were generated
by a process called "subtractive immunization" in the
following manner (see also Brooks et al., *J. Cell. Biol.*
122: 1351-1359, 1993).

10 C57 BL6 female mice, 6-8 weeks old and 16-18
grams, were obtained from Charles River (Montreal,
Quebec). On day 1, subconfluent cultures of M⁻ HEp-3
cell line were washed three times with sterile PBS and
harvested by incubation with 3 ml of nonenzymatic cell
15 dissociation solution (Sigma Chem. Co., St. Louis, MO)
for 1 min. Cells were resuspended in sterile PBS (4 x
10⁶ cells/ml) and 2 x 10⁶ cells were inoculated i.p. into
mice. On days 2 and 3, mice were injected i.p. with 200
mg/kg cyclophosphamide (Sigma Chem. Co., St. Louis, MO)
in sterile PBS. As a tolerizing (immunosuppressive)
20 agent, Cyclophosphamide suppressed the immune response
towards dominant immunodeterminants present on the tumor
cells. On day 15, sera were collected and analyzed in
the whole cell ELISA assay for reactivity for M⁻ HEp-3
cells. On day 18, M⁺ HEp-3 cells were harvested in the
25 same manner as described above for M⁻ HEp-3 cells and 2 x
10⁶ cells were inoculated i.p. into the mice. On day 39,
mice received an i.p. boost of 3 x 10⁶ M⁺ HEp-3 cells.
On day 42, sera were collected and analyzed for
reactivity toward the M⁺ HEp-3 cells in the whole cell
30 ELISA. On day 43, the mouse with the greatest M⁺/M⁻ ratio
was sacrificed for the production of hybridomas.
Variations of the above-described subtractive
immunization (S.I.) protocol included the time

cyclophosphamide was given, either at 120 and 96 h before the M⁻HEp-3 cells or 1 and 24 h after the M⁻HEp-3 cells. A control protocol was performed that was exactly the same as the S.I. protocol except that no cyclophosphamide was given.

Hybridomas were produced according to the standard methods (Worthington et al., *Br. J. Haematol.* 74: 216, 1990). Briefly, the spleens and accessory lymph nodes were removed, washed and fused (4:1 spleen to myeloma) with murine myeloma cell line NSO. The cells were plated (2.5×10^4 cells/0.1 ml HAT medium/well) in 96 well tissue culture plates (Falcon, Becton Dickinson Labware). Culture supernatants were screened by the whole cell ELISA and hybridomas from each of the immunization protocols were cloned by soft agar and limiting dilution. Each positive hybridoma was cloned twice. The hybridomas producing antibodies which recognized M⁺ cells over M⁻ cells by a factor of 2-fold or higher were selected for further characterization and testing in the metastasis assay.

Monoclonal antibodies were purified from the hybridoma conditioned media as follows. The hybridoma conditioned medium was centrifuged at 3,000 g for 30 min, concentrated 10 X with an Amicon Stirred Cell Concentrator (Amicon, Beverly, MA) and filtered through a 0.22- μ m filter (Acrodisc, Gelman Sciences, Ann Arbor, MI). The concentrated conditioned medium was applied to a protein G Sepharose column (Pharmacia LKB Biotechnology, Piscataway, NJ) connected to a FPLC (Pharmacia LKB Biotechnology). MAbs were eluted with 0.1 M glycine (pH 3.0). Purified mAbs were immediately

neutralized with 1.0 M Tris, pH 9.0, dialyzed against PBS and stored at -20°C.

EXAMPLE 3

IDENTIFICATION OF THE TARGET ANTIGENS

The Target Antigen of mAb 41-2

5 mAb 41-2 was used in a large-scale
immunoprecipitation to isolate sufficient amounts of the
antigen from Triton X-100 lysates of HEp-3 cells for
amino acid sequencing. The immunoprecipitate was
resolved by SDS-PAGE under reducing conditions, and
10 following transfer of the resolved proteins to Immobilon-
P, a Ponceau-stainable band was observed at 180 kDa (the
mobility of the 180 kDa metastasis-associated antigen
does not change under reducing conditions). In duplicate
immunoblots, the 180 kDa protein was immunostained with
15 mAb 41-2, but not with normal mouse IgG or with secondary
antibody alone. The immunopurified metastasis-associated
antigen was subjected to Edman degradation and a partial
amino acid sequence, FEIALPRESQITVLQ(I)KXGT (SEQ ID NO:
3) was obtained from the amino terminus (residue #15 is
20 either Q or I; residue 17 has not been determined).
Comparison of this partial amino acid sequence with known
sequences in the NCBI database revealed that this antigen
has not been previously cloned.

25 The Target Antigen of mAbs 50-6 and 1A5

mAbs were used to screen a HEp-3 cDNA
expression library in the following manner. Highly
metastatic HEp-3 cells were generated by continuous
passage on the CAM. Primary HEp-3 cell cultures were
30 established from the metastatic CAM tumors as described
by Testa (Cancer Res. 52: 5597-5603, 1992). The
cultured HEp-3 cells were trypsinized and passaged at

confluence. After two weeks of culture *in vitro*, poly A+ RNA was isolated from the monolayer HEp-3 cells using the FastTrack mRNA isolation system (Invitrogen) according to the manufacturer's instructions, and stored at -80°C. At the same time, cells from a parallel culture plate were tested in the CAM assay system to confirm their metastatic behavior prior to construction of the cDNA library.

A custom-made M+ HEp-3 cDNA library was constructed in the eukaryotic expression vector pcDNA I (Invitrogen Corp., San Diego, CA) using the poly A+ RNA isolated as described above. pcDNA I is a plasmid useful for expression cloning in eukaryotic cells. pcDNA I contains the SV40 and polyoma origins of replication which permit episomal replication when transfected into eukaryotic cells expressing the large T antigen (e.g., COS monkey cells), thus allowing high copy numbers of the introduced cDNAs in the cells and accumulation of high levels of the encoded antigens. In addition, the encoded proteins are glycosylated by the COS cells and directed to their normal subcellular location.

First strand synthesis from the HEp-3 RNA was primed with a Not "T" primer, a modified oligo dT primer with an internal Not I site which allowed unidirectional cloning of the cDNA into BstX/NotI-cut pcDNA I. A library size of 3.84×10^6 primary recombinants was obtained. Of 10 randomly picked clones, all contained inserts. The average insert size was 1.5 kb, with a range of 1.0 kb to 3.1 kb.

A portion of the library was grown in liquid culture. Plasmid DNA was purified with a QIAGEN Plasmid Kit (QIAGEN) following the manufacturer's instructions.

Such purified plasmid DNA was used for transfection into COS cells. Twenty-four hours prior to transfection, 0.7×10^6 COS cells were plated in 100 mm dishes. The cells were transfected with the plasmid DNA (10 μ g/dish) using the calcium phosphate method (Wigler et al., *Proc. Natl. Acad. Sci. USA* 76: 1373-1376, 1979) with a glycerol shock 6 hours after application of the DNA. Seventy-two hours after transfection, the cells were detached from the plates with Non-enzymatic Cell Dissociation Solution (Sigma) and washed twice in calcium- and magnesium-free PBS containing 10 mM EDTA and 5% FBS (PBS/EDTA/FBS). The cells were resuspended in PBS/EDTA/FBS containing 0.4 μ g/ml of mAb 50-6, and incubated on ice for 1 hr. The suspension was centrifuged, the supernatant was removed, and the cells were washed once in PBS/EDTA/FBS.

COS cells expressing the HEp-3 antigen recognized by mAb 50-6 were isolated by immunological panning according to the method of Wysocki and Sato (*Proc. Natl. Acad. Sci. USA* 75: 2844-2848, 1978). Briefly, antibody-incubated cells were resuspended in PBS/EDTA/FBS and 3 ml of the suspension was added to a 100 mm bacteriological dish previously coated with goat anti-mouse IgG. The plates were incubated on a level surface at room temperature for 2 hr, after which the plates were gently washed with 3 x 10 ml of PBS/EDTA/FBS. Because of the presence of EDTA in the solution, attachment of the COS cells with the panning plate was mediated via antibody interactions. Episomal plasmid DNA was recovered from the selected COS cells by Hirt lysis (Hirt, *J. Mol. Biol.* 26: 365-369, 1967), and rescued by electroporation into highly competent bacteria. The resulting bacterial colonies were scraped off the plates

and grown in liquid culture, from which culture plasmids were isolated with the QIAGEN Plasmid Kit. COS cells were subjected to a second round of transfection, incubation with mAb 50-6, panning, Hirt lysis and plasmid rescue as described above. Bacterial transformants were expanded in culture and the plasmids isolated for a third round of transfection. Plasmids were rescued from the transfected COS cells, amplified in bacterial culture and size selected on an agarose gel. COS cells were transfected with the size-selected plasmid fractions and immunostained with mAb 50-6. One positive fraction was identified. Bacteria were transformed with plasmids isolated from this fraction, and mini-prep plasmid DNA was isolated from individual colonies. COS cells were transfected with the individual plasmid DNA preparations and immunostained with mAb 50-6 in order to identify the clone encoding the antigen recognized by the antibody. A positive clone was identified and the insert was sequenced (**Figure 1A**). The open reading frame (**SEQ ID NO: 1**) of this clone encodes a core protein (**Figure 1B**, **SEQ ID NO: 2**) having a predicted molecular mass of 27.8 kD. There are four distinct hydrophobic domains, and a single N-glycosylation site in a large hydrophilic region that separates the third and fourth hydrophobic domains. Comparison of the sequence of this clone with the NCBI database identified the metastasis-associated antigen as PETA-3/CD151, a recently described member of the transmembrane 4 superfamily (TM4SF) of proteins, which are also known as tetraspanins. The coding sequence was identical to that reported by Fitter, et al. (*Blood* 8: 1348-1355, 1995).

The cell surface distribution of PETA-3 on HEp-3 cells and COS cells transfected with the cloned cDNA was analyzed by immunocytochemistry. On HEp-3 cells incubated with mAb 50-6, there was a strong staining pattern over the entire surface of the cell membrane, including filopods extending from the cell body (**Figure 2a**). The same cell surface staining pattern was seen on COS cells transiently transfected with PETA-3 cDNA (**Figure 2c**). No signal was observed on HEp-3 or PETA-3-transfected COS cells incubated with normal mouse IgG (**Figures 2b and 2d**), or on control COS transfectants incubated with mAb 50-6 (**Figure 2e**).

Lysates of Hep-3 cells and PETA-3-transfected COS cells were analyzed by Western Blot using mAbs 50-6 and 1A5. As shown in **Figure 3A**, the PETA-3-encoded protein co-migrates with the 29 kD antigen expressed on HEp-3 cells. As shown in **Figure 3B**, mAb 1A5 recognized PETA-3/CD151 expressed by the COS cells (lane 3), whereas no signal was observed in lysates from control transfectants (lane 2). A minor 25 kDa band, immunostained with mAbs 50-6 and 1A5, was also evident in lysates of transfected COS cells (**Figures 3A and 3B**) and in HEp-3 lysates (visible upon prolonged exposure of the Western blot). This 25 kDa band is likely the non-glycosylated form of the protein, since cultivation of HEp-3 cells with tunicamycin or treatment of HEp-3 cell lysates with N-glycanase generates a 25 kDa band that is immunoreactive with mAb 1A5.

These results demonstrate that both mAb 50-6 and mAb 1A5 recognize PETA-3.

EXAMPLE 4
EXPRESSION OF THE TARGET ANTIGENS

Expression of the Target Antigen of mAb 41-2

5 Cell lines were examined for expression of the target antigen of mAb 41-2 by whole cell ELISA as described in Example 1. Positive staining by mAb 41-2 was observed with human epidermoid carcinoma HEp-3 cells as well as several human breast carcinoma cell lines. 10 Among the human breast carcinoma cell lines examined (**Figure 4**), MDA-MB-231 cells showed the highest immunoreactivity; immunostaining of MDA-MB-468 cells was about 32% that of the MDA-MB-231 cells; and there was little reactivity with MDA-MB-361 cells (about 6% of that 15 seen with MDA-MB-231). The metastatic potential of these three breast carcinoma cells lines has been quantitated and ranked by Zhang et al. (*Invasion and Metastasis* 11: 204-215, 1991) as follows: MDA-MB-231 > MDA-MB-468 > MDA-MB-361, from highest to lowest. Thus the 20 immunoreactivity of these cells with mAb 41-2 correlated with their relative metastatic potential.

 The expression of the target antigen of mAb 41-2 in normal human mammary epithelial cells or HMEC (primary cell culture from Clonetics Corp., San Diego, 25 CA) was compared with the expression in MDA-MB-231 cells or HEp-3 cells in a Whole Cell ELISA assay. As shown in **Figure 5**, the level of expression of the target antigen in HMEC was 2.7-fold lower than that in the metastatic human breast carcinoma cell line MDA-MB-231.

30 The antigen expressed by MDA-MB-231 cells comigrates with the HEp-3 antigen on Western Blots (**Figure 6**).

Expression of the Target Antigen of mAb 50-6

PETA-3 was found to be expressed by HeLa cells, HT1080 cells (human fibrosarcoma line), M24met cells (human melanoma line), and MDA-MB-231 cells (human breast carcinoma cell line). In addition, when compared to normal human mammary epithelial cells, the level of PETA-3 expression by the MDA-MB-231 cells is ten times higher (Figure 7).

EXAMPLE 5**INHIBITION OF TUMOR CELL METASTASIS
BY mAb 41-2, mAb 50-6 AND mAb 1A5**

5 The effects of the mAbs on tumor metastasis
were examined using human epidermoid carcinoma HEP-3
cells in a chick embryo metastasis assay system. Both
spontaneous metastasis and experimental metastasis were
analyzed.

Spontaneous Metastasis Assays

10 A small window was cut through the shell with
the use of a small model grinding wheel (Dremel, Division
of Emerson Electric Co., Racine, WI). HEP-3 cells (2 x
15 10⁵ in 25 µl) were inoculated through the window onto the
surface of the underlying chorioallantoic membranes
(CAMs) of 10-day old chick embryos. The windows were
sealed with sterile tape, and the embryos were returned
to the incubator. After 24 hours, a second window was
20 cut in the side of the eggshell directly over a prominent
blood vessel, which was located by candling the egg. The
shell membrane was made transparent with a drop of
paraffin oil and the purified mAb (200 µg per embryo in
100 µl of PBS) was inoculated into the vessel using a 1
25 cc syringe fitted with a 30 ga needle. Control embryos
received the same amount of normal mouse IgG. The second
window was sealed and the embryos were incubated for
another 6 days. The eggs were then opened and the CAM
tumors were excised and weighed as a measure of
30 tumorigenesis. The lungs of the chick embryos were also
removed, finely minced and passaged onto the CAMs of a
second group of embryos. These embryos were incubated
for additional seven days to allow any HEP-3 cells in the

lungs to multiply. The "lung tumors" arising from the transferred lungs were then excised, finely minced and the number of HEp-3 cells were identified using a phase contrast microscope. Five randomly selected microscopic fields were counted and the number of HEp-3 cells were recorded. The lung tumors were also assayed biochemically for HEp-3 cells by quantitating human uPA activity.

Human uPA activity was measured in the following manner. Lung tumor minces were placed on ice, homogenized with a tissue grinder (Biospec Products, Bontlesville, OK) in 0.9 ml of extraction buffer (25 mM Tris, 0.5% TX-100, pH 8.1), and insoluble material was removed by centrifugation at 10,000 g for 10 min. The concentrations of protein in the lung tumor extracts were determined by the BCA method (Pierce). Lung tumor extracts at a protein concentration of 0.1 mg/ml were assayed for human uPA activity. The uPA assays were performed as described by Friberger (*Haemostasis* 7: 138-145, 1975) in 96 well flat bottom ELISA plates (NUNC, Roskilde, Denmark). Lung tumor extracts (10 μ l of the 0.1 mg/ml) were incubated with purified human plasminogen (3 μ g/well) for 2 h at 37°C. The S2251 chromogenic substrate (Kabi Vitrum, Stockholm, Sweden) was added (15 μ g/3 μ l extraction buffer) to each well and the ELISA plates were incubated at 37°C for 0, 15, 30, 60 and 90 min. The absorbance at 405 nm was read at each time point with a multiscan plate reader. The human uPA activity was calculated by comparison of the sample values with a standard curve derived from human urokinase standards (Leo Pharmaceutical Products, Denmark). uPA

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activity was calculated as milli units of human uPA activity per mg of lung protein.

Measurement of human uPA and visual scoring provided simple, reproducible and quantitative tests for the metastatic spread of HEP-3 cells in the chick embryo. It was found that MAb 41-2 inhibited spontaneous HEP-3 metastasis by about 63.7% (**Table I**), MAb 50-6 inhibited spontaneous HEP-3 metastasis by about 74% (**Table II**), and 1A5 inhibited spontaneous HEP-3 metastasis by about 93%.

Experimental Metastasis Assay

A prominent blood vessel was located in the CAM of 11-day old chicken embryos. A window was cut into the shell over the vessel and the underlying shell membrane was made transparent with a drop of paraffin oil. The tumor cell suspension ($2 \times 10^5/\text{ml}$) was incubated with the mAb (2 mg/ml) for 5 minutes at room temperature, then 100 μl of the cell/antibody suspension was inoculated directly into the circulatory system using a 1 cc syringe fitted with a 30 ga needle. Control embryos were inoculated with tumor cells pre-incubated with normal mouse IgG. The window was sealed with a piece of sterile tape and the embryos were incubated for additional 6 days. The assay was then completed as described above for spontaneous metastasis assays.

The results showed that mAb 41-2 inhibited experimental metastasis by about 90.8% (**Table I**), mAb 50-6 inhibited experimental metastasis by about 56.8% (**Table II**) and mAb 1A5 inhibited experimental metastasis by about 81%.

TABLE I. Inhibition of HEp-3 metastasis by mAb 41-2.

Spontaneous metastasis				
antibody	number of embryos	primary tumor wt.* (mean \pm SEM)	Lung metastasis+ (huPA in lung) (mean \pm SEM)	% inhib
NM IgG	9	222 \pm 26.2	4287 \pm 1078	0
mAb 41-2	11	203 \pm 23.3	1556 \pm 437	63.70%
Experimental metastasis				
antibody	number of embryos		Lung metastasis ⁺⁺ (huPA in lung) (mean \pm SEM)	% inhib
NM IgG	10		3855 \pm 1025	0
mAb 41-2	11		333 \pm 132	90.80%

*P=0.300; +P=0.019; ++P=0.003

TABLE II. Inhibition of HEp-3 metastasis by mAb 50-6.

Spontaneous metastasis				
antibody	number of embryos	primary tumor wt.* (mean \pm SEM)	Lung metastasis+ (huPA in lung) (mean \pm SEM)	% inhib
NM IgG	11	274 \pm 51	1015 \pm 249	0
mAb 50-6	13	253 \pm 46	261 \pm 117	74.30%
Experimental metastasis				
antibody	number of embryos		Lung metastasis ⁺⁺ (huPA in lung) (mean \pm SEM)	% inhib
NM IgG	11		2169 \pm 601	0
mAb 50-6	10		937 \pm 299	56.80%

*P=0.689; +P=0.022; ++P=0.044

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PETA-3/CD151 mediates an early event in the formation of metastatic foci.

The anti-metastatic properties of mAb 50-6 was not due to a cytostatic or cytotoxic effect of the antibody, since it was found that the antibody had no effect on proliferation of HEP-3 cells *in vitro*, nor on the size of the primary tumor on the CAM (**Table II**).

A time-course study of inhibition of experimental metastasis was carried out. The antibodies were inoculated at different times before or after inoculation of the tumor cells. The inoculated embryos were incubated for an additional six days, after which the lungs were excised, finely minced and transferred to prepared CAMs of a second set of embryos. The assay was then completed as described above. As shown in **Table III**, HEP-3 dissemination to the embryonic lungs was markedly inhibited when mAb 1A5 was administered as early as two hours before or as late as six hours after inoculation of the tumor cells. In contrast, there was little effect on HEP-3 colonization of the lungs when mAb 1A5 was administered 10 or 20 hours after the tumor cells were inoculated. The inability of mAb 1A5 to inhibit lung colonization at the latter time points (when tumor cells would likely have extravasated already) indicates that the antibody did not block metastasis by inhibiting cell growth at the secondary sites.

These experiments indicate that PETA-3/CD151 mediated HEP-3 dissemination by affecting an earlier event such as cell adhesion to the vessel wall, extravasation, and/or tumor cell migration to selective sites of secondary growth.

TABLE III. Time Course of inhibition of HEp-3 experimental metastasis.

antibody	time of Ab inoculation	number of embryos	Lung metastasis (huPA in lung) (mean \pm SEM)	% inhib
NM IgG	- 2hr	47	1522 \pm 54	0
mAb 1A-5	- 2hr	36	295 \pm 60	81
mAb 1A-5	+ 6hr	21	401 \pm 36	74
mAb 1A-5	+10hr	8	1471 \pm 36	3
mAb 1A-5	+20hr	20	1408 \pm 67	7

EXAMPLE 6**INHIBITION OF TUMOR CELL INVASION BY mAb 41-2**

Matrigel-coated invasion chambers (Biocoat Invasion
5 Chambers, 24-well format) were purchased from
Collaborative Research Products and prepared (rehydrated)
according to the manufacturer's instructions. Tumor cell
suspensions were prepared with Non-enzymatic Cell
Dissociation Solution (Sigma). MAb (50 µg/ml) was
10 included in the upper chamber with the tumor cells (1×10^4 /well) and in the lower reservoir which contained NIH-
3T3 conditioned medium as a chemoattractant. Control
chambers received normal mouse IgG (50 µg/ml). The
chambers were incubated at 37°C in 5% CO₂ and, after 70
15 hr, the cells on the upper surface of the filters were
removed by wiping with a cotton swab. The filters were
then fixed with methanol and stained with hematoxylin and
eosin, and the number of cells per microscopic field were
counted from the underside of the filter. **Figure 8**
20 graphically depicts such invasion assay.

As shown in **Figure 9**, mAb 41-2 inhibited Matrigel
invasion by both HEp-3 cells and MDA-MB-231 cells. MAb
41-2 inhibited invasion of MDA-MB-231 cells by 38%, and
inhibited invasion of HEp-3 invasion by 63%, when
25 compared to the control chambers. Under identical
conditions, mAb 41-2 had no effect on invasion by HT1080,
a human fibrosarcoma cell line (American Type Culture
Collection, 12301 Parklawn Drive, Rockville, Maryland,
20852-1776) which was not recognized by this antibody.

EXAMPLE 7**INHIBITION OF TUMOR CELL
MIGRATION BY mAb 41-2, mAb 50-6 AND mAb 1A5**

5 Control invasion chambers (no coating on the filter, 24-well format) were purchased from Collaborative Research Products. mAb (50 µg/ml), together with the tumor cells (2×10^4 /well), was added in the upper chamber. mAb (50 µg/ml) was also added in the lower
10 reservoir having serum-containing medium as a chemoattractant. In control chambers, normal mouse IgG (50 µg/ml) was used in place of mAb. The chambers were incubated at 37°C in 5% CO₂. After 18 hr, the cells on the upper surface of the filters were removed by wiping
15 with a cotton swab. The filters were then fixed with methanol and stained with hematoxylin and eosin, and the number of migrated cells per microscopic field was recorded from the underside of the filter. **Figure 10** graphically depicts such migration assay.

20 It was found that mAb 41-2 inhibited HEp-3 migration by approximately 50%. mAb 50-6 and mAb 1A5 inhibited HEp-3 migration by approximately 45% and 44%, respectively, when compared to controls ($P=0.034$) (**Figure 11**).

25 To further demonstrate the role of PETA-3/CD151 in tumor cell migration, HeLa cells were transfected with the cloned cDNA, or with vector alone. Two clones from each group were selected on the basis of the PETA-3 expression levels, the similar rates of growth *in vitro*
30 and similarity in morphological appearance. The levels of cell-surface PETA-3 on the two over-expressing clones,

PB6_{HI} and PC3_{HI} were about two-fold higher than the HEp-3 cells, while the control transfectants, NB11_{LO} and NB17_{LO} expressed approximately one quarter to one half the amount detected on HEp-3 cells (**Figure 12A**). The growth rates of the four clones were identical (**Figure 12B**). Migration by the over-expressing clones PB6_{HI} and PC3_{HI} was significantly higher than the underexpressing control transfectants NB11_{LO} and NB17_{LO} (**Figure 12C**; P=0.001). When mAb 50-6 or mAb 1A5 (50 ug/ml) was added to the chambers, migration of clone PB6_{HI} was significantly reduced (P=0.034) by 51.6% and 52.8%, respectively, while migration of clone PC3_{HI} was significantly reduced (P=0.007) by 36.7 % and 50.6%, respectively, lowering the motility of these cells to the level of control NB11_{LO} cells. Neither mAb 50-6 nor mAb 1A5 inhibited migration of the weakly expressing clones NB11_{LO} (P=0.729) or NB17_{LO} (P=0.337), indicating that migration by these cells was mediated by a PETA-3-independent mechanism.

EXAMPLE 8
Association of PETA-3 with Integrin $\alpha_3\beta_1$

5 A monoclonal antibody, mAb 1992, which specifically recognizes the $\alpha_3\beta_1$ heterodimer, was used in whole cell ELISA. The $\alpha_3\beta_1$ integrin was detected on HEp-3 cells (**Figure 13**) at levels equivalent to known positive controls, MDA-MB-231, a human breast carcinoma cell line, and HT1080, a human fibrosarcoma cell line.

10 HEp-3 cell lysates were immunoprecipitated with mAb 1992 as follows. HEp-3 cells were lysed in Brij lysis buffer (1% Brij 98, 25 mM HEPES, pH 7.5, 150 mM NaCl, 5mM MgCl_2 , 10 μM E64, 20 U/ml Aprotinin, 20 $\mu\text{g/ml}$ soybean trypsin inhibitor (all from Sigma)) for one hr with constant rocking at 4°C. Insoluble material was removed by centrifugation at 12,000 g for 5 minutes at 4°C. The lysate was pre-cleared with GammaBind Plus Sepharose beads. Aliquots of the extract representing 10^7 cells were incubated with either 25 μg of mAb 1A5, 10 μg of mAb 1992 (Chemicon, Temecula, CA), or 25 μg of normal mouse IgG (Sigma, St. Louis, MO). Fifty μl of packed GammaBind Plus Sepharose beads were added to each sample and the mixtures were incubated at 4°C overnight with constant rocking. The beads were then washed with the Brij lysis buffer, and the immune complexes were eluted with Laemmli sample buffer at 95°C for two minutes. The eluted proteins were resolved on a 10% SDS-PAGE gel, transferred to nitrocellulose. Because biotinylation of both mAb 1A5 and mAb 50-6 destroys their immunoreactivity, PETA-3 was detected by incubating the blots with unlabeled mAb 1A5

followed by horseradish peroxidase-conjugated goat anti-mouse IgG.

Western blot analysis of immunoprecipitates obtained with mAb 1992 revealed a 29 kD protein which
5 immunostained with mAb 1A5 (**Figure 14**, lane 3). This protein co-migrated with the authentic antigen present in the HEP-3 lysates (**Figure 14**, lane 1) and in immunoprecipitates obtained with mAb 1A5 (**Figure 14**, lane 2). No PETA-3 signal was observed in control normal
10 mouse IgG immunoprecipitates probed with mAb 1A5 (**Figure 14**, lane 4) or normal mouse IgG (**Figure 14**, lane 5). The background bands observed in the immunoprecipitated samples are due to the reactivity of the secondary antibody to mouse IgG proteins and IgG fragments as seen
15 in the control lanes.

When the PETA-3-transfected HeLa clones were analyzed by whole-cell ELISA, there was no detectable $\alpha_3\beta_1$ signal. Weak signals were seen with the control HeLa transfectant (**Figure 13**). This indicates that PETA-3
20 interacts with a different integrin in the transfected HeLa cells to effect the observed increase in cell migration (**Figure 12C**).

EXAMPLE 9
EFFECTS OF THE MONOCLONAL ANTIBODIES
ON CELL ADHESION

5 HEp-3 cells and the HeLa transfectants were tested
for adhesion to various purified matrix proteins in the
presence of mAb 50-6, mAb 1A5 or normal mouse IgG. HEp-3
cells, PETA-3-overexpressing HeLa clones (PB6_{HI}, PC3_{HI}) and
control HeLa clones (NB11_{LO}, NB17_{LO}) were added to the
10 wells of 96-well plates coated with either fibronectin
(FN) or vitronectin (VN) in the presence of 50µg/ml mAb
50-6, mAb 1A5 or normal mouse IgG and incubated for 30
minutes. Non-adherent cells were gently washed away, and
the amount of cell adhesion was determined by crystal
15 violet staining. Data are expressed as the optical
density (OD) at 595 nm of the incorporated crystal violet
dye, and are representative of two separate experiments
conducted in triplicate.

20 As shown in **Figures 15A-B**, at the 30 minute time
point, HEp-3 attachment to both FN (**Figure 15A**) and VN
(**Figure 15B**) was unaffected by the presence of either of
the anti-metastatic mAbs. Likewise, attachment of the
over-expressing and weakly expressing HeLa transfectants
to FN or VN was not blocked by mAb 50-6 or mAb 1A5.
25 There was also no correlation between levels of PETA-3
expression and cell attachment. Similar results were
obtained when the adhesion assays were terminated after
15 minutes, and when cell attachment was tested on plates
coated with laminin, type I collagen and type IV
30 collagen. These data indicate that PETA-3 is not
functionally involved in cell attachment.

 HEp-3 cells were tested for adhesion to Fibronectin
(FN) in the presence of mAb 41-2 or normal mouse IgG.

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HEp-3 cells were added to the wells of 96-well plates coated with fibronectin in the presence of 50µg/ml mAb 41-2 or normal mouse IgG, and incubated for various times as indicated. Non-adherent cells were gently washed
5 away, and the amount of cell adhesion was determined by crystal violet staining. Data are expressed as the optical density (OD) at 595 nm of the incorporated crystal violet dye.

10 As shown in **Figures 15C**, the inhibition of the HEp-3 attachment to FN by mAb 41-2 was 100% at the 10 minute time point, was about 53% at the 20 minute time point, and was about 38% at the 30 minute time point.

EXAMPLE 10
INHIBITION OF GROWTH-FACTOR
INDUCED ANGIOGENESIS BY mAb 50-6

5 Collagen/mesh rafts which were used for angiogenesis
assays were prepared in the following manner. Each raft
was composed of an experimental or control collagen
solution overlaid upon a single small nylon mesh placed
10 above a single larger mesh. Nylon meshes (Tetko Nitex 3-
180/43) were cut to 4 mm by 4 mm (hereafter referred to
as "bottom mesh") and 2 mm by 2 mm (hereafter referred to
as "top mesh") and sterilized by autoclaving. Purified
type I collagen (3.1 mg/ml; Vitrogen) was neutralized on
ice with addition of 10X phosphate-buffered saline (PBS),
15 1 N sodium hydroxide, and 1 M HEPES. Experimental or
control solutions were prepared in PBS, including basic
fibroblast growth factor at a final concentration of 16
 μ g/ml and bovine serum albumin at a final concentration
of 1 mg/ml and kept on ice. Normal mouse IgG or mAb 50-6
20 were added to collagen solutions at a final concentration
of either 50 or 100 μ g/ml. Final collagen solutions were
pipetted onto meshes, and rafts were placed in a 37.5°
humidified incubator. After incubation for 45 minutes,
rafts were ready for use in angiogenesis assays.

25 Just laid, pathogen-free fertile White Leghorn eggs
were obtained from Spafas, Inc. and placed in a 42°C
humidified incubator on day 0. Sterile dishes and lids
were prepared. Eggs were removed from the incubator on
day 3, wiped with ethanol and shell contents were emptied
30 into dishes using sterile technique. Thereafter, chicken
embryos were maintained in a 37.5° humidified cell
culture incubator. On day 8, embryos were removed from
the incubator, collagen/mesh rafts prepared as described

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above were added. Each set of experiment typically included three control rafts (no growth factor, growth factor alone and growth factor together with the control antibody) and one experimental raft (growth factor plus experimental antibody). One of each raft was placed on the exposed chorioallantoic membrane of a separate 8 day-old chicken embryo. Embryos were returned to the incubator for an additional 64-66 hours (through day 11).

On day 11, embryos were removed from the incubator and examined under a Wild Heerbrugg M3 stereomicroscope at 90X magnification. The presence or absence of blood vessels at or above the plane of the top mesh was assessed for each box in the grid defined by the top mesh. The angiogenesis score was expressed as the fraction of boxes in top mesh containing vessels out of the total number of boxes in the top mesh. **Figure 16** graphically depicts the angiogenesis assay.

Basic fibroblast growth factor (bFGF) stimulates angiogenesis on the CAM (**Figures 17-18**). However, the formation of new blood vessels is reduced by 55% in the presence of mAb 50-6 (**Figure 18**). Control antibody (normal mouse IgG) had no effect on vessel formation. These results indicated that mAb 50-6 inhibited the function of its target antigen expressed by embryonic cells, and blocked the ability of these cells to form new vessels in response to bFGF.

EXAMPLE 11**POTENTIAL FUNCTIONAL DOMAINS/EPITOPES OF PETA-3**

Both mAb 50-6 and mAb 1A5 were anti-functional and thus, it is proposed that the epitope(s) recognized by these antibodies represent a functional domain(s). Since neither mAb recognized the mouse homologue of PETA-3 (**Figure 19**), the amino acid sequences of the mouse (**SEQ ID NO: 4**) and human (**SEQ ID NO: 2**) proteins were aligned to identify differences in the human sequence. As seen in **Figure 20**, the small extracellular loop (amino acids 40-57, double underlined) contains only a single substitution, while in the large extracellular loop (amino acids 113-221, double underlined) there are a number of differences. Most of the changes are conservative. However, there are seven non-conservative substitutions (shaded in gray).

The epitope(s) recognized by mAb 50-6 and mAb 1A5 are conformational, since neither antibody recognized PETA-3 on Western blots of gels run under reducing conditions. The non-conservative substitutions in human PETA-3 could alter the conformation of the molecule relative to the mouse homologue, and are candidates for mutagenesis in order to identify the epitope(s) and/or functional domains of the protein.

The amino acid sequence of the large extracellular loop of human PETA-3 was also compared to human CD9 and CD63, two tetraspanins known to suppress metastasis (Hotta et al., *Melanoma Res.* 1: 125-132, 1991; Ikeyama et al., *J. Exp. Med.* 177: 1231-1237, 1993; Radford et al., *In. J. Cancer* 62: 631-635, 1995). As seen in **Figure 21**, four of the seven non-conserved amino acid residues (P¹³⁷,

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G¹⁷⁶, G¹⁷⁷ , and Q¹⁹⁴) identified in the comparison of mouse and human PETA-3 are absent in both of the other human tetraspanins, while the remaining three (Q¹⁴⁸ , R¹⁶⁵ , Q¹⁷³) are either missing or not well conserved in at least one of the other molecules. The alignment shown in **Figure 21** also shows that the overall homology between the large extracellular loops of these three proteins is poor and there are many regions of the PETA-3 molecule that would be appropriate for mutagenesis studies.

5
10

WHAT IS ClaimED IS:

1. A hybridoma cell line 41-2 (ATCC #).
- 5 2. A hybridoma cell line 50-6 (ATCC #).
3. A monoclonal antibody 41-2.
4. A monoclonal antibody 50-6.
- 10 5. A functional derivative of mAb 41-2.
6. A functional derivative of mAb 50-6.
- 15 7. An isolated antigen recognized by mAb 41-2.
8. The antigen of Claim 7, wherein said antigen is associated with tumor metastasis, having a Mw of about 180 kd on SDS gel under reducing conditions and
20 comprising a partial amino acid sequence of SEQ ID NO: 3.
9. An antibody directed towards the antigen of Claim 7, wherein said antibody is monoclonal or polyclonal.
- 25 10. A polyclonal antibody directed against the PETA-3 antigen.
11. An isolated nucleic acid molecule, wherein said
30 nucleic acid molecule encodes the antigen of Claim 8.

12. An expression vector comprising the isolated nucleic acid molecule of Claim 11.

5 13. A host cell transformed with the expression vector of Claim 12.

10 14. A pharmaceutical composition comprising a pharmaceutical carrier and the PETA-3 antigen, a portion of PETA-3, the antigen recognized by mAb 41-2 or a portion thereof.

15 15. A pharmaceutical composition comprising a pharmaceutical carrier and at least one of an antibody directed against PETA-3 or an antibody directed against the target antigen of mAb 41-2.

20 16. The pharmaceutical composition of Claim 14, wherein said antibody is one of mAb 41-2, mAb 50-6, mAb 1A-5, or a functional derivative thereof.

25 17. A method of diagnosing a metastatic tumor in a subject, comprising detecting the expression of at least one of the PETA-3 antigen or the antigen recognized by mAb 41-2, and determining a substantial increase of the expression of the antigen in the tissue being diagnosed compared with normal tissue.

30 18. The method of Claim 17, wherein the expression is detected by using an antibody or a nucleic acid probe.

19. The method of Claim 18, wherein said antibody is mAb 41-2, mAb 50-6, mAb 1A-5, or a functional derivative thereof.

5 20. A method of treating a metastatic tumor in a subject, comprising administering to the subject a therapeutically effective amount of a tumor metastasis-inhibiting antibody directed against PETA-3.

10 21. The method of Claim 20, wherein said antibody is mAb 50-6, mAb 1A-5, a functional derivative of mAb 50-6, or a functional derivative of mAb 1A-5.

15 22. A method of treating a metastatic tumor in a subject, comprising administering to the subject a therapeutically effective amount of an antisense molecule of the PETA-3 gene.

20 23. A method of treating a metastatic tumor in a subject, comprising administering to the subject a therapeutically effective amount of a tumor metastasis-inhibiting antibody directed against or the target antigen of mAb 41-2.

25 24. The method of Claim 23, wherein said antibody is mAb 41-2 or a functional derivative thereof.

30 25. A method of treating a metastatic tumor in a subject, comprising administering to the subject a therapeutically effective amount of an antisense molecule of the gene encoding the target antigen of mAb 41-2.

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26. A method of treating an aberrant PETA-3-
dependent angiogenesis in a subject, comprising
administering to the subject a therapeutically effective
amount of an angiogenesis-inhibiting antibody directed
5 against PETA-3 or a functional derivative thereof.

[illegible]

FIGURE 1A

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* * * SEQUENCE * * *

GGAGCTTCTG TCCACCTGTC CTGCAGAGGA GTCGTTTCCA GCCCGGCTGC CCCAGGATG 59
M

GGT GAG TTC AAC GAG AAG AAG ACA ACA TGT GGC ACC GTT TGC CTC AAG 107
G E F N E K K T T C G T V C L K

TAC CTG CTG TTT ACC TAC AAT TGC TGC TTC TGG CTG GCT GGC CTG GCT 155
Y L L F T Y N C C F W L A G L A

GTC ATG GCA GTG GGC ATC TGG ACG CTG GCC CTC AAG AGT GAC TAC ATC 203
V M A V G I W T L A L K S D Y I

AGC CTG CTG GCC TCA GGC ACC TAC CTG GCC ACA GCC TAC ATC CTG GTG 251
S L L A S G T Y L A T A Y I L V

GTG GCG GGC ACT GTC GTC ATG GTG ACT GGG GTC TTG GGC TGC TGC GCC 299
V A G T V V M V T G V L G C C A

ACC TTC AAG GAG CGT CGG AAC CTG CTG CGC CTG TAC TTC ATC CTG CTC 347
T F K E R R N L L R L Y F I L L

CTC ATC ATC TTT CTG CTG GAG ATC ATC GCT GGT ATC CTC GCC TAC GCC 395
L I I F L L E I I A G I L A Y A

TAC TAC CAG CAG CTG AAC ACG GAG CTC AAG GAG AAC CTG AAG GAC ACC 443
Y Y Q Q L N T E L K E N L K D T

ATG ACC AAG CGC TAC CAC CAG CCG GGC CAT GAG GCT GTG ACC AGC GCT 491
M T K R Y H Q P G H E A V T S A

GTG GAC CAG CTG CAG CAG GAG TTC CAC TGC TGT GGC AGC AAC AAC TCA 539
V D Q L Q Q E F H C C G S N N S

CAG GAC TGG CGA GAC AGT GAG TGG ATC CGC TCA CAG GAG GCC GGT GGC 587
Q D W R D S E W I R S Q E A G G

CGT GTG GTC CCA GAC AGC TGC TGC AAG ACG GTG GTG GCT CTT TGT GGA 635
R V V P D S C C K T V V A L C G

CAG CGA GAC CAT GCC TCC AAC ATC TAC AAG GTG GAG GGC GGC TGC ATC 683
Q R D H A S N I Y K V E G G C I

ACC AAG TTG GAG ACC TTC ATC CAG GAG CAC CTG AGG GTC ATT GGG GCT 731
T K L E T F I Q E H L R V I G A

GTG GGG ATC GGC ATT GCC TGT GTG CAG GTC TTT GGC ATG ATC TTC ACG 779
V G I G I A C V Q V F G M I F T

TGC TGC CTG TAC AGG AGT CTC AAG CTG GAG CAC TAC TGA CCC TGC CTT 825
C C L Y R S L K L E H Y

FIGURE 1B

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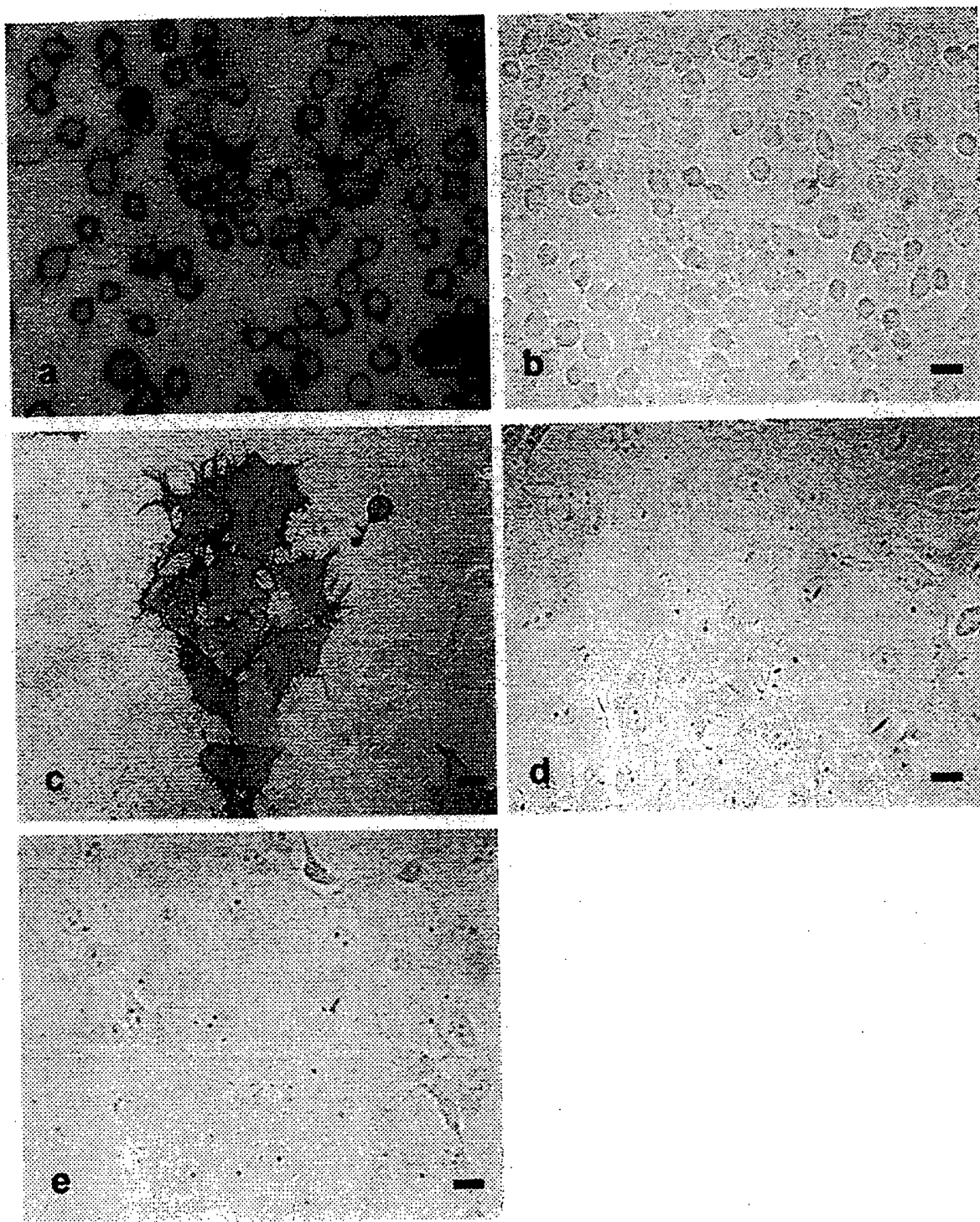


FIGURE 2

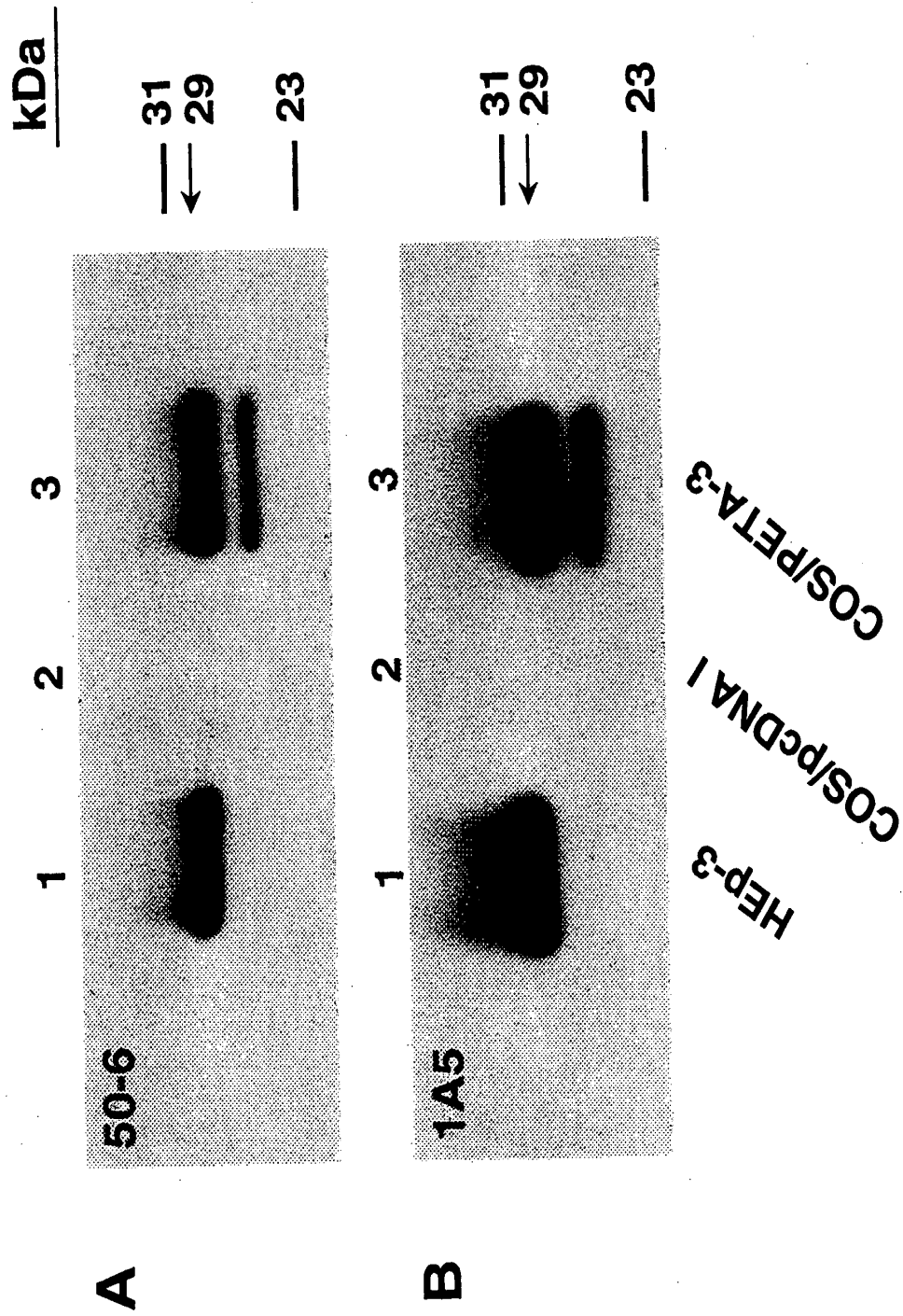


FIGURE 3

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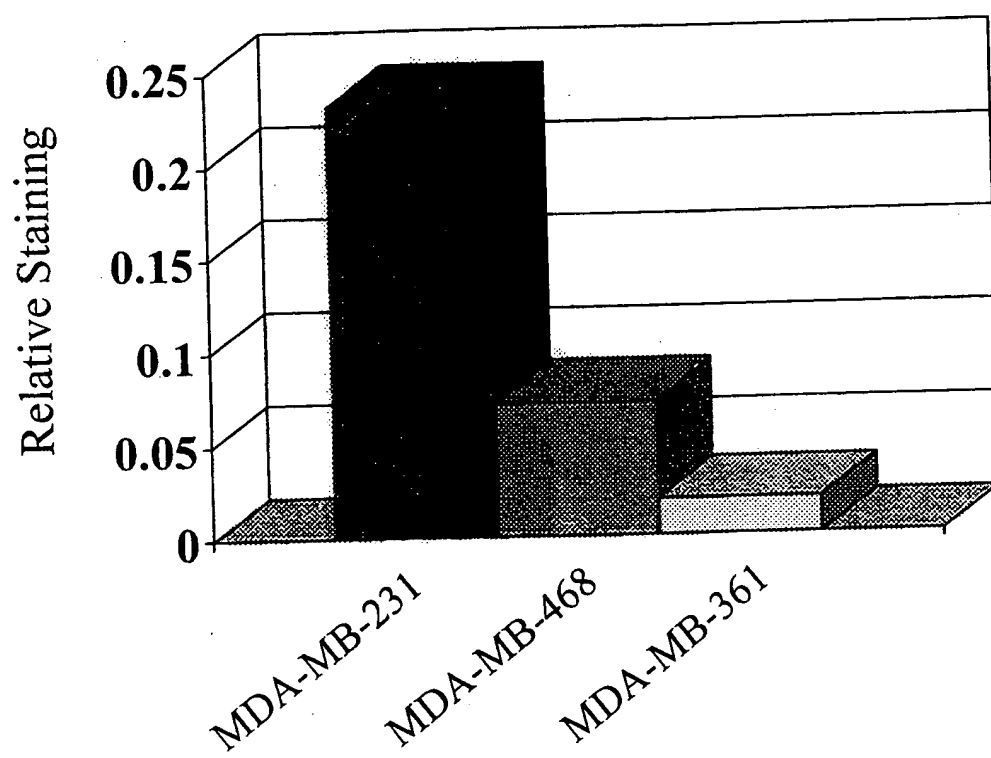


FIGURE 4

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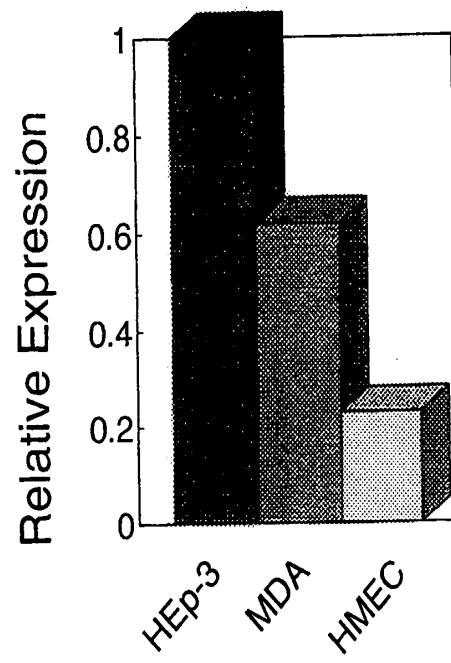


FIGURE 5

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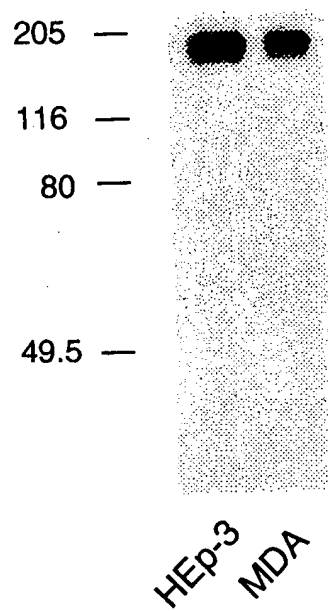


FIGURE 6

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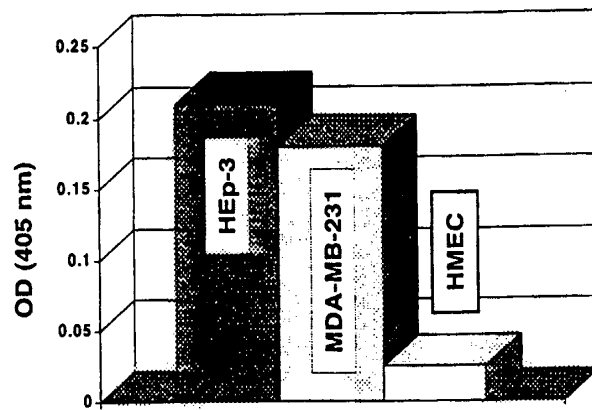


FIGURE 7

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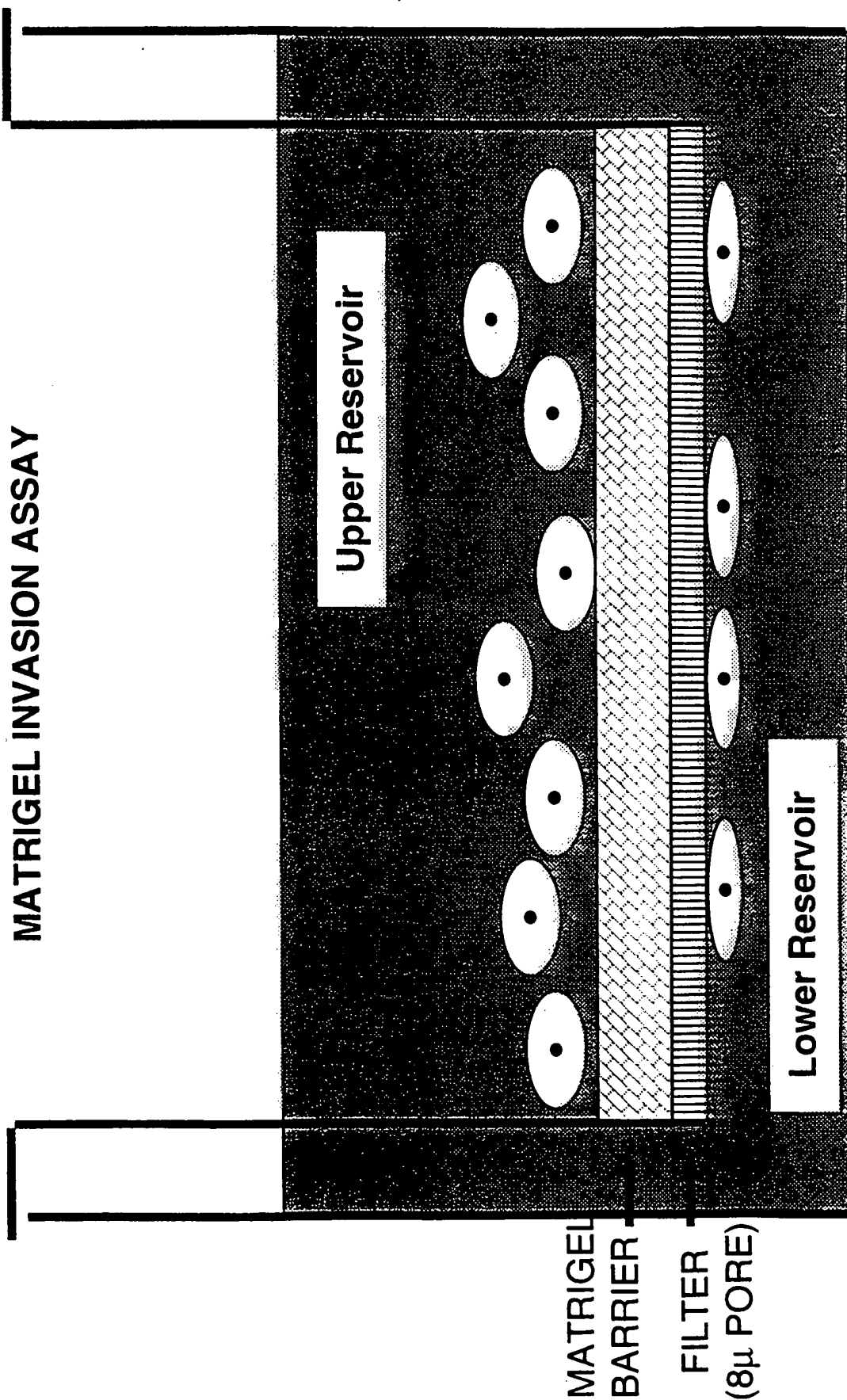


FIGURE 8

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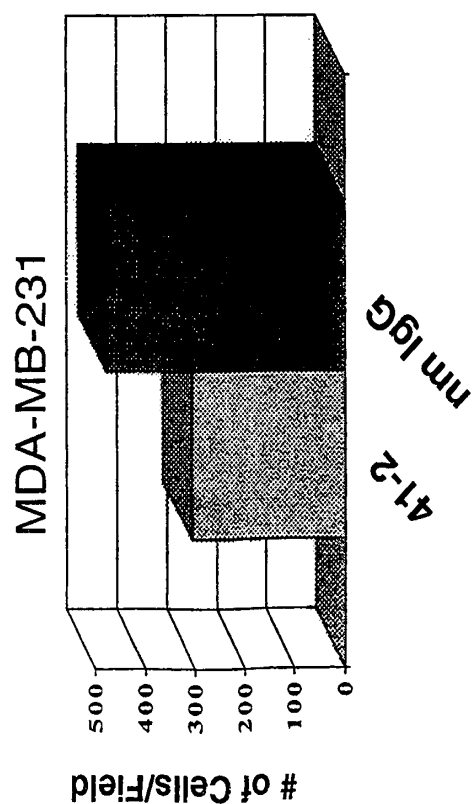
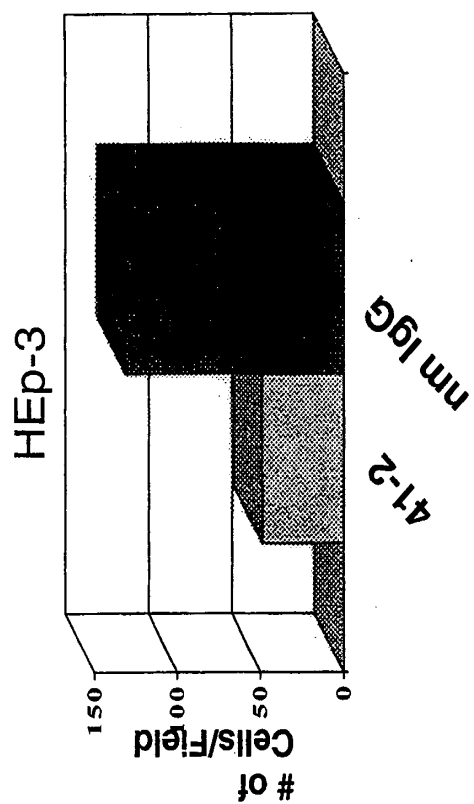


FIGURE 9

MIGRATION ASSAY

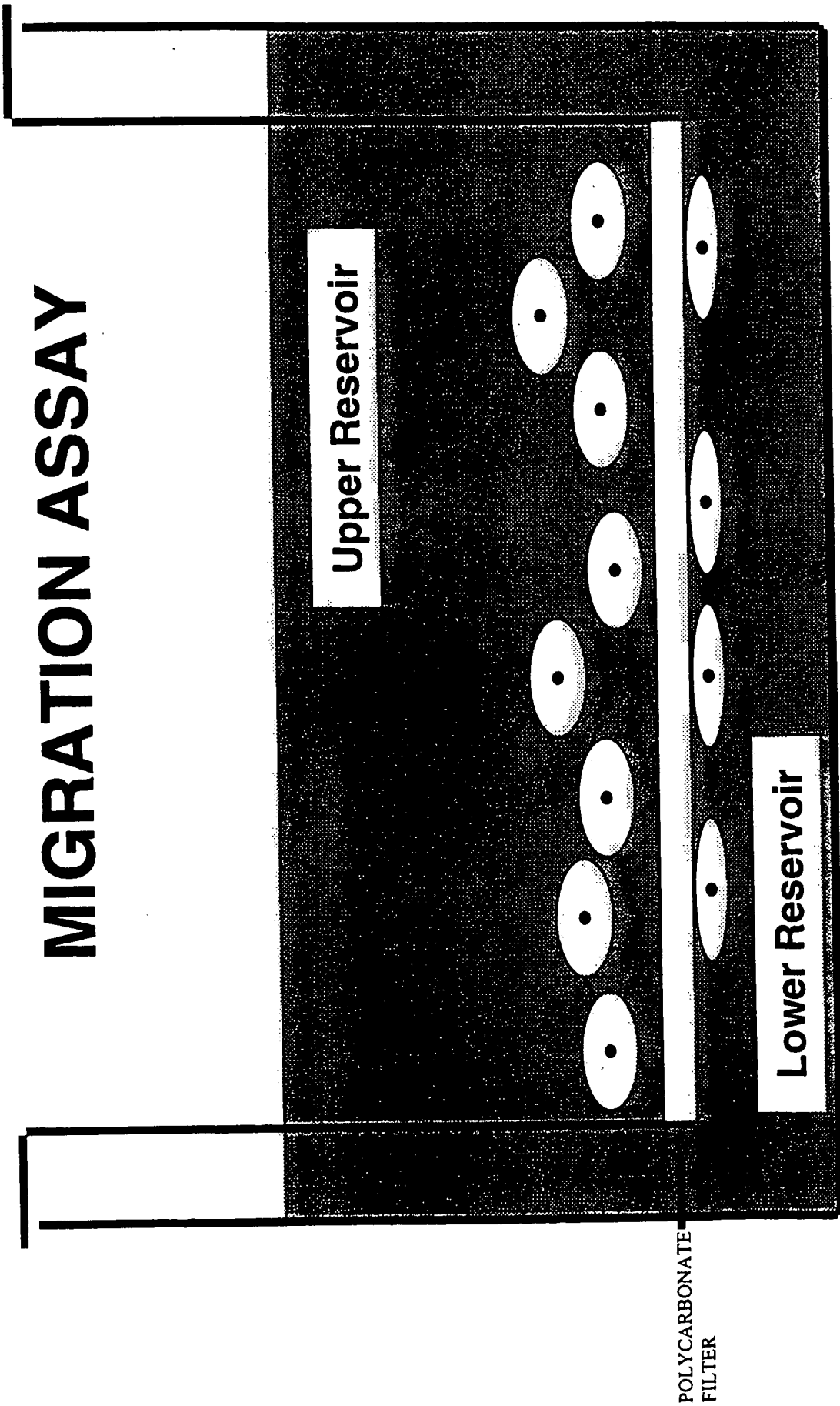


FIGURE 10

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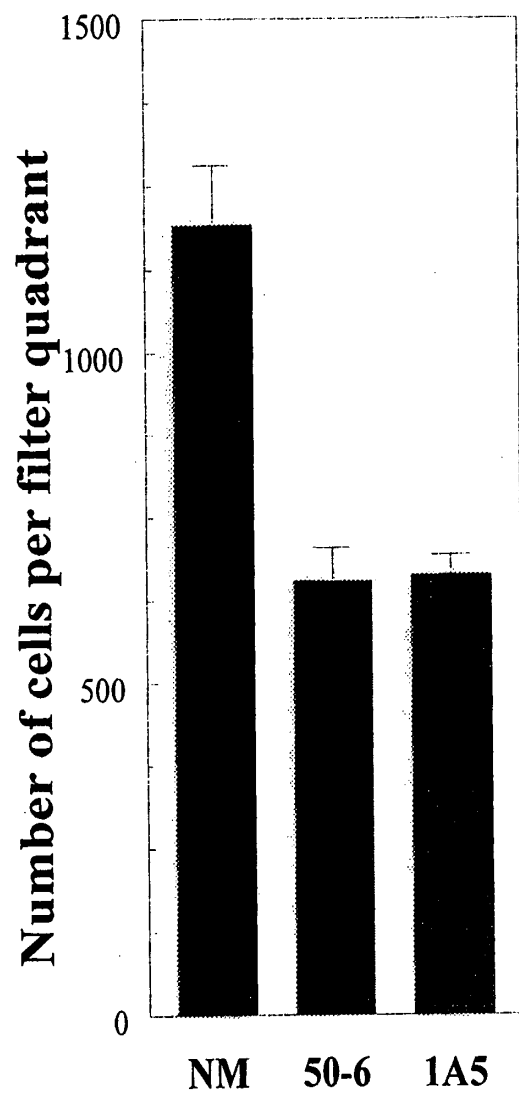


FIGURE 11

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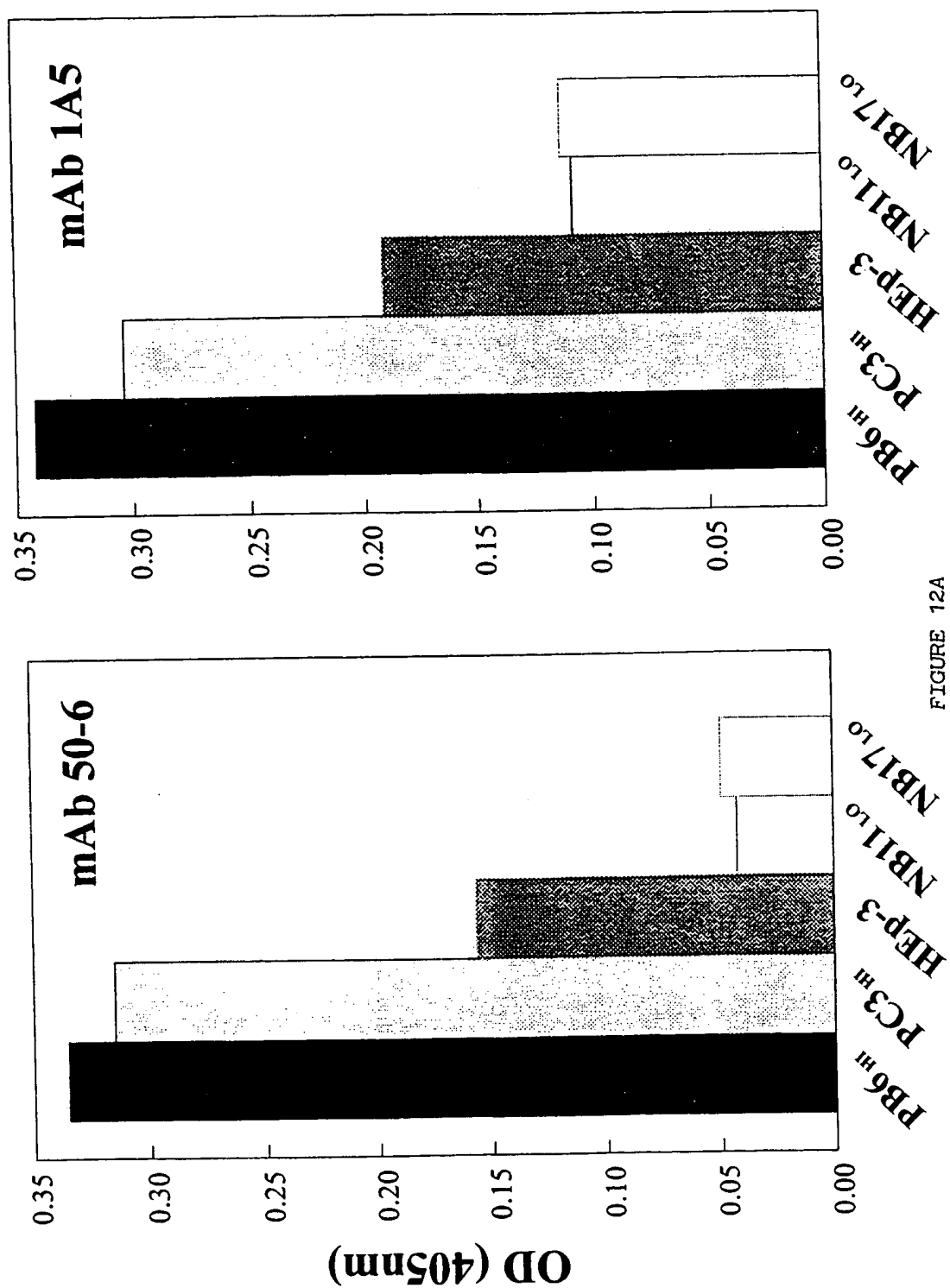


FIGURE 12A

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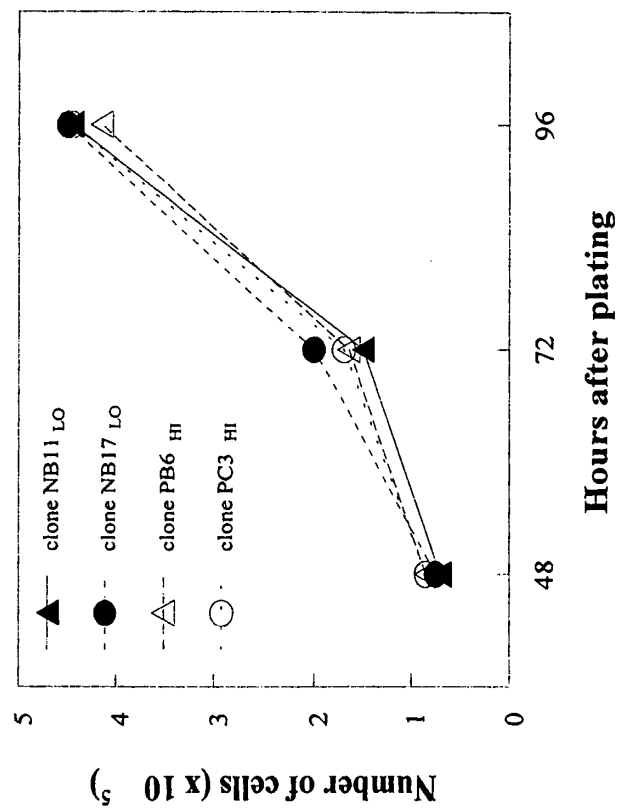
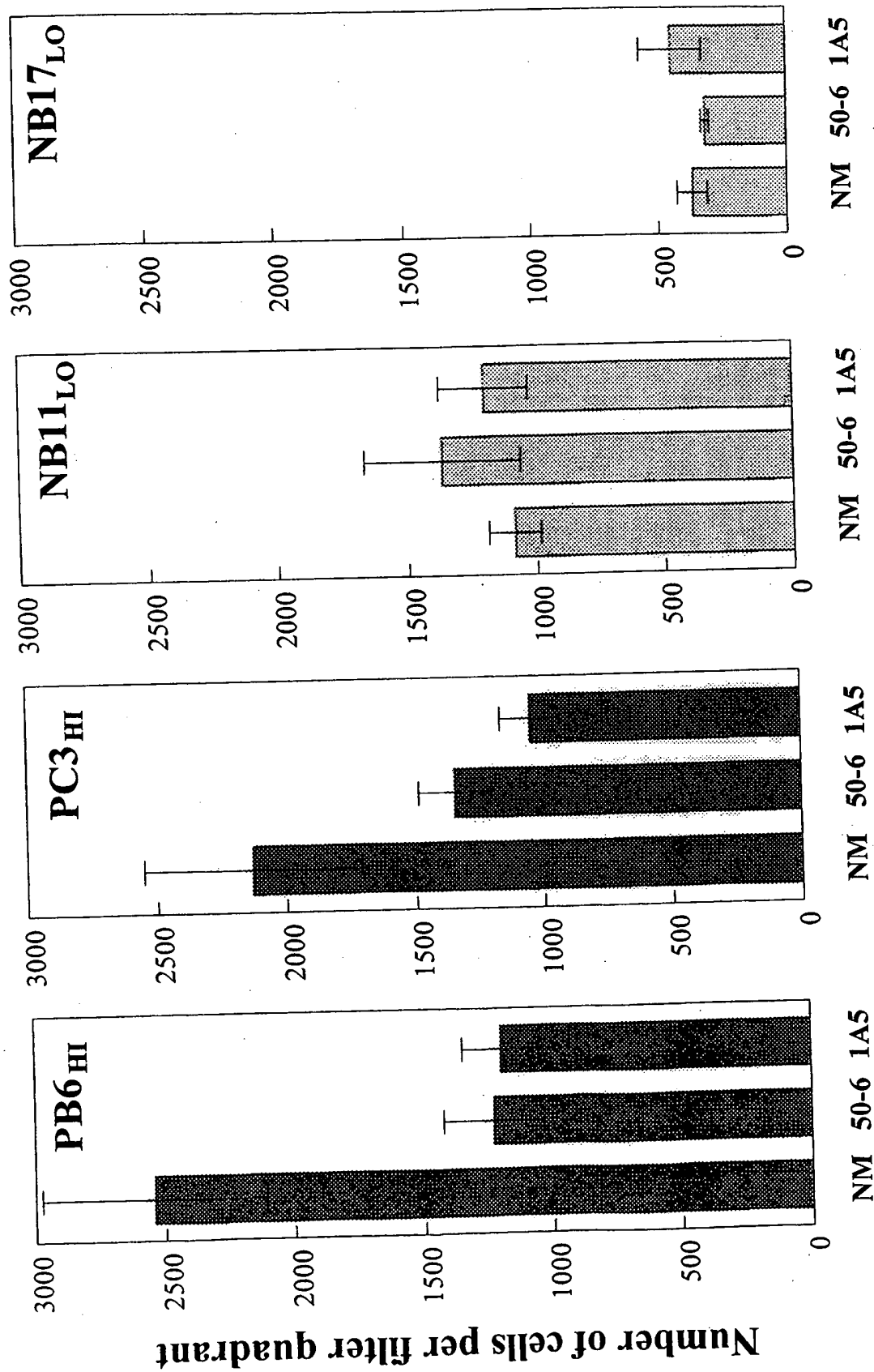


FIGURE 12B

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CONTROL CLONES

PETA-3 OVER-EXPRESSORS

FIGURE 12C

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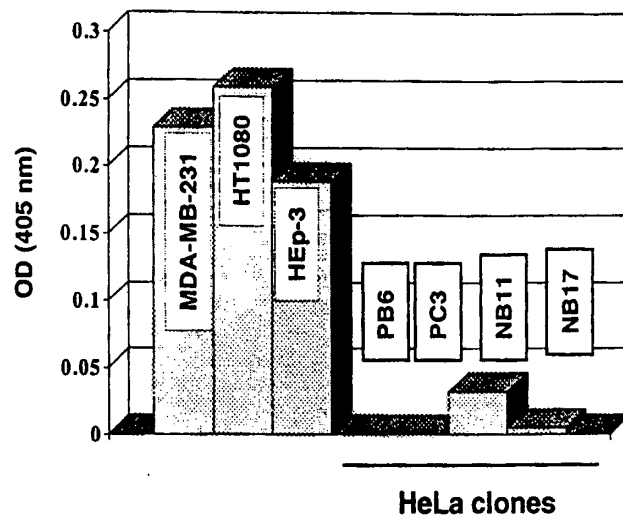


FIGURE 13

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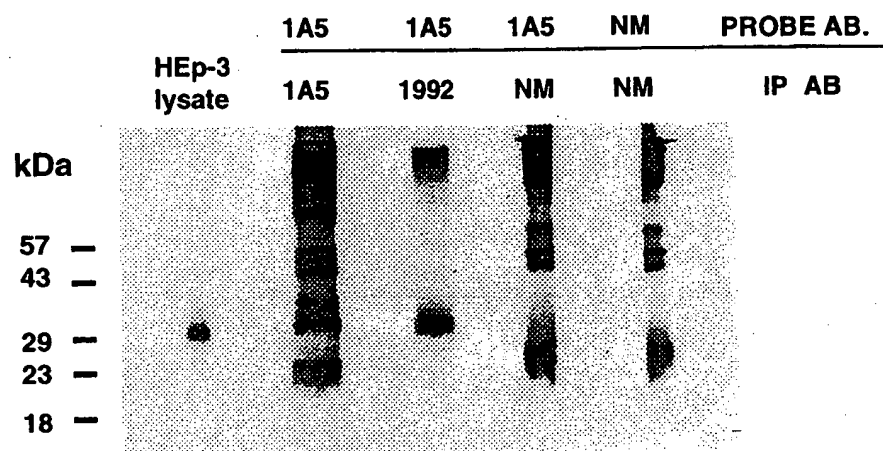


FIGURE 14

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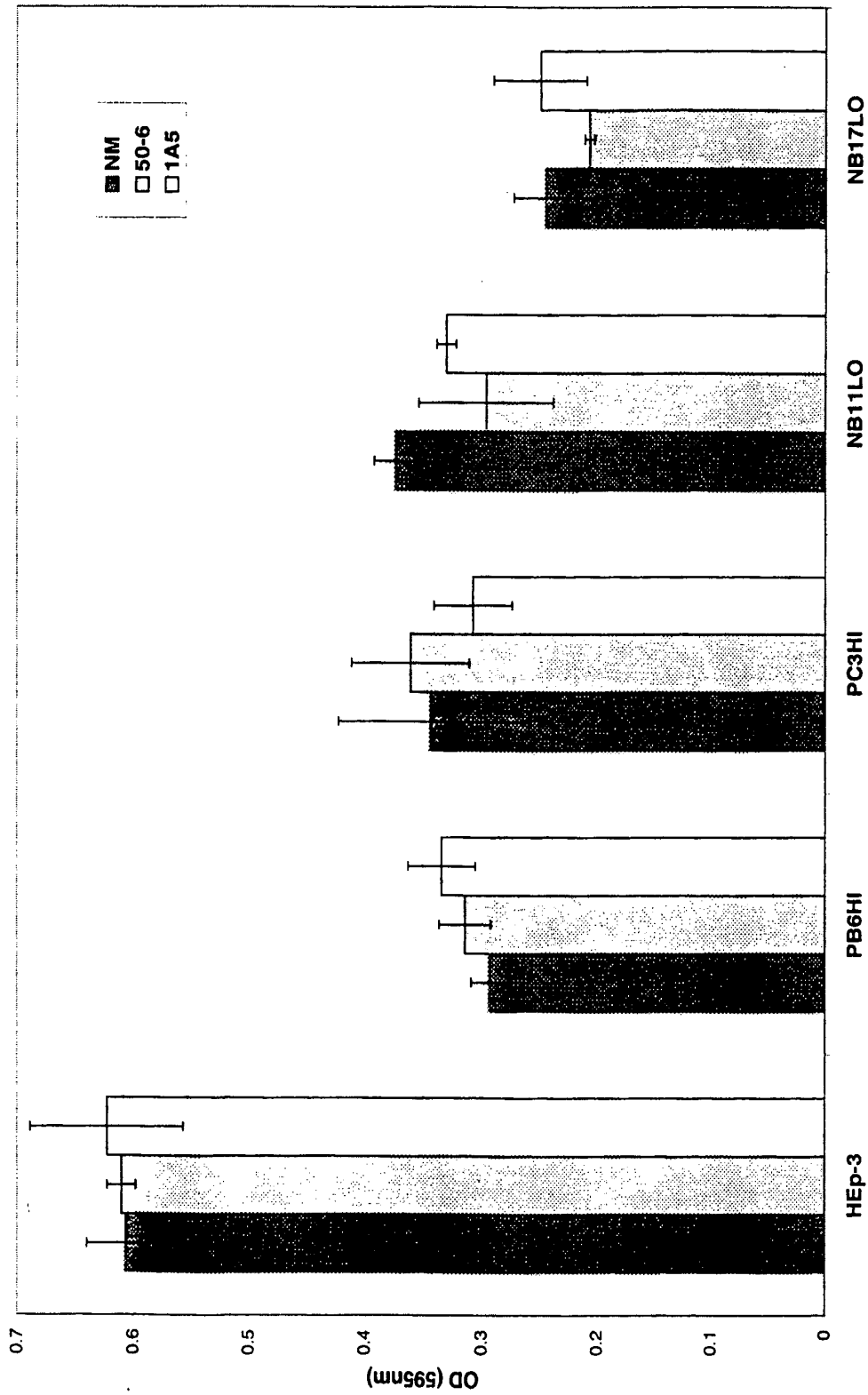


FIGURE 15A

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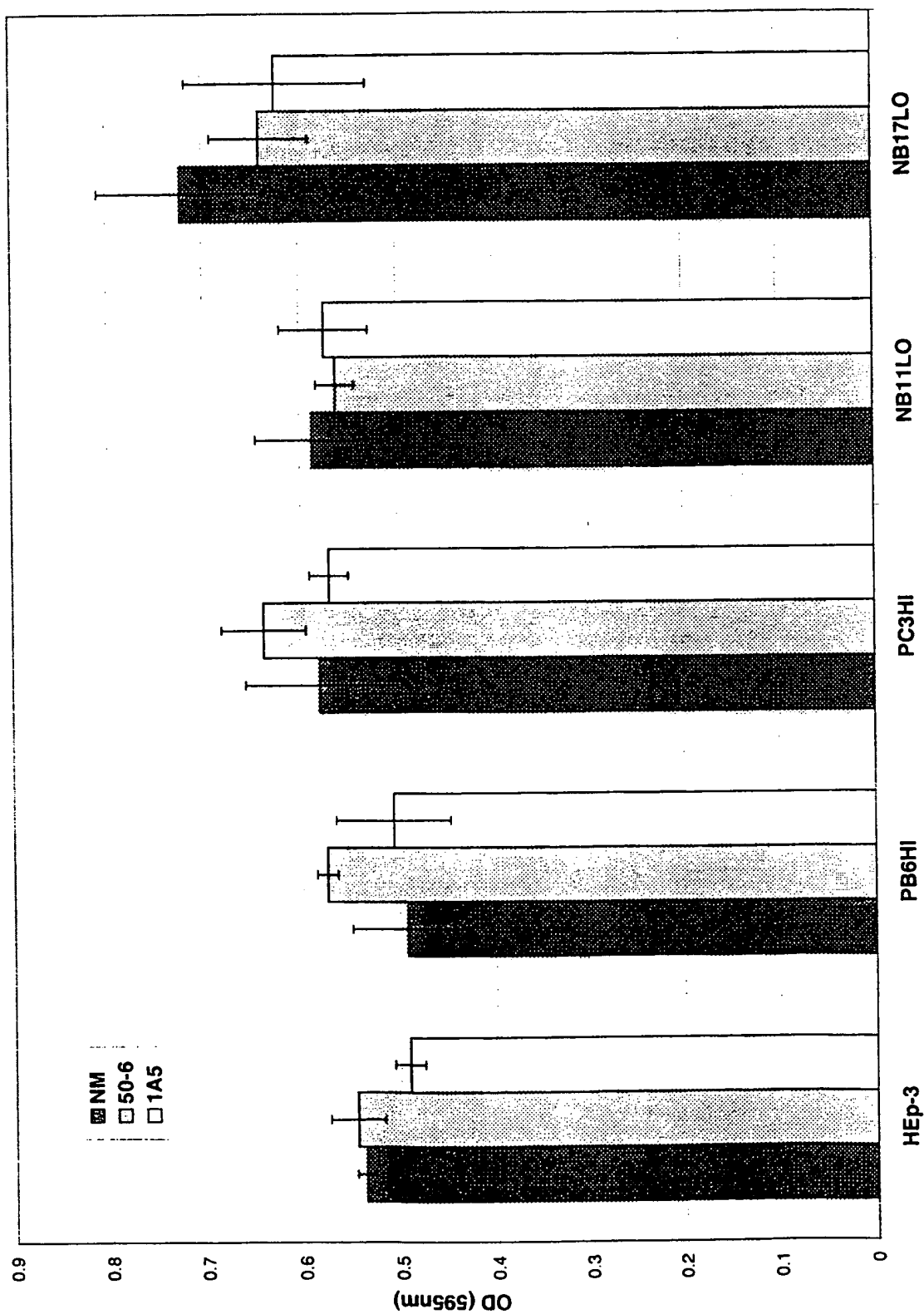


FIGURE 15B

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Inhibition of Hep-3 cell attachment to fibronectin

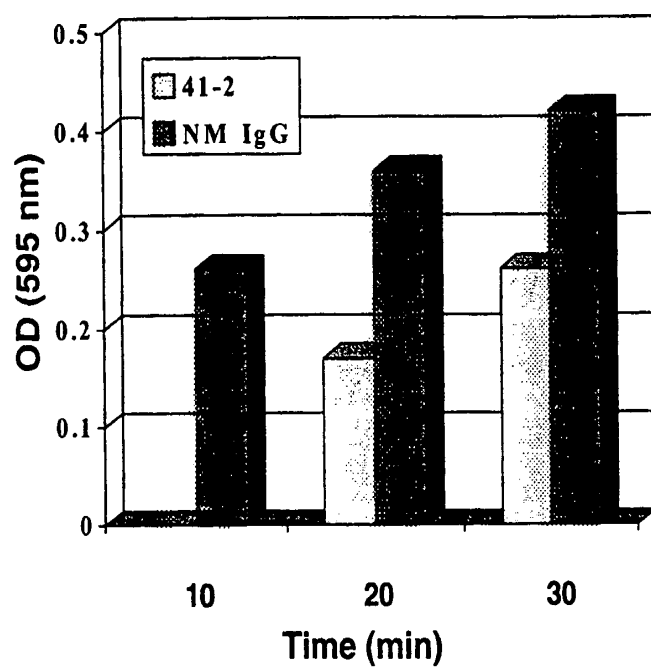
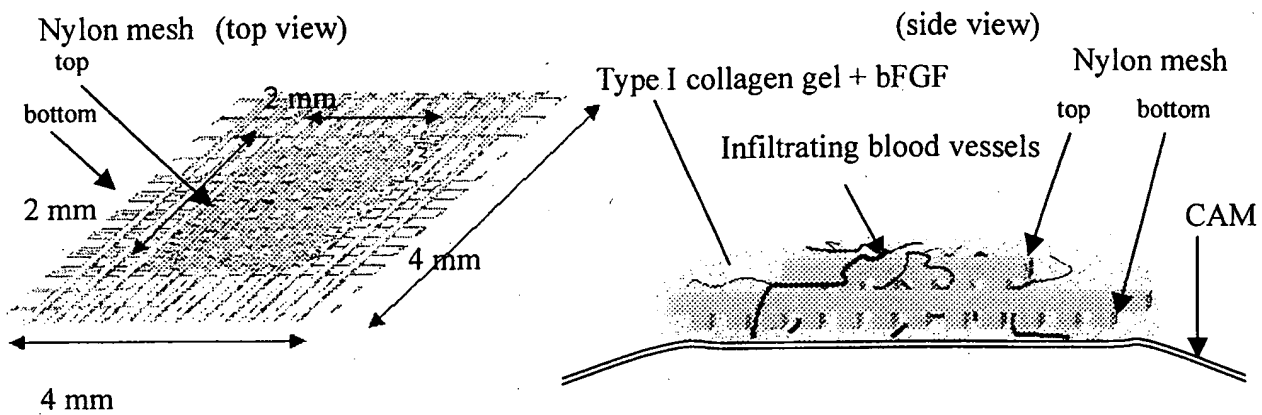


FIGURE 15C

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Type I collagen solution applied to two layers of nylon mesh and placed on CAM



Views of collagen/mesh raft upon CAM of day 11 chick embryo
(low power) (high power)

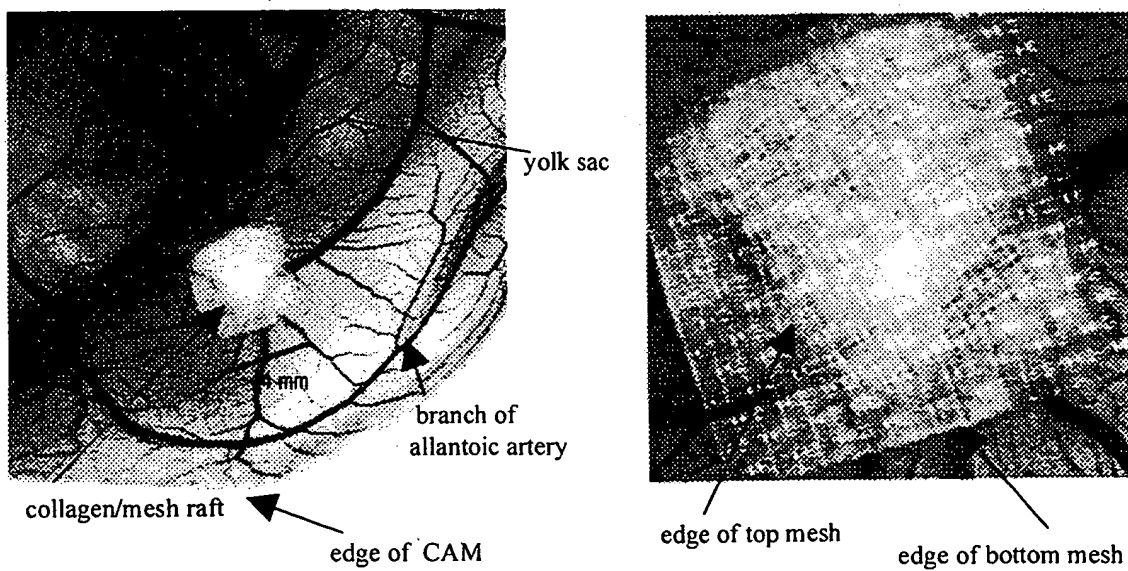


FIGURE 16

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Effect of bFGF on neovascularization of type I collagen matrix:
light microscopic view

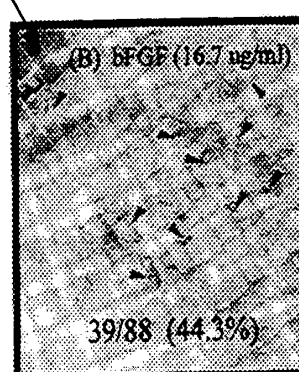
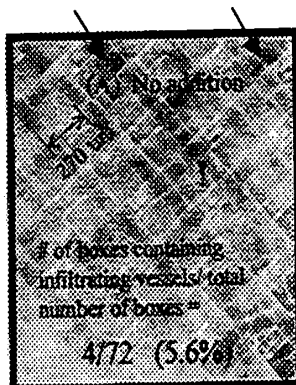


FIGURE 17

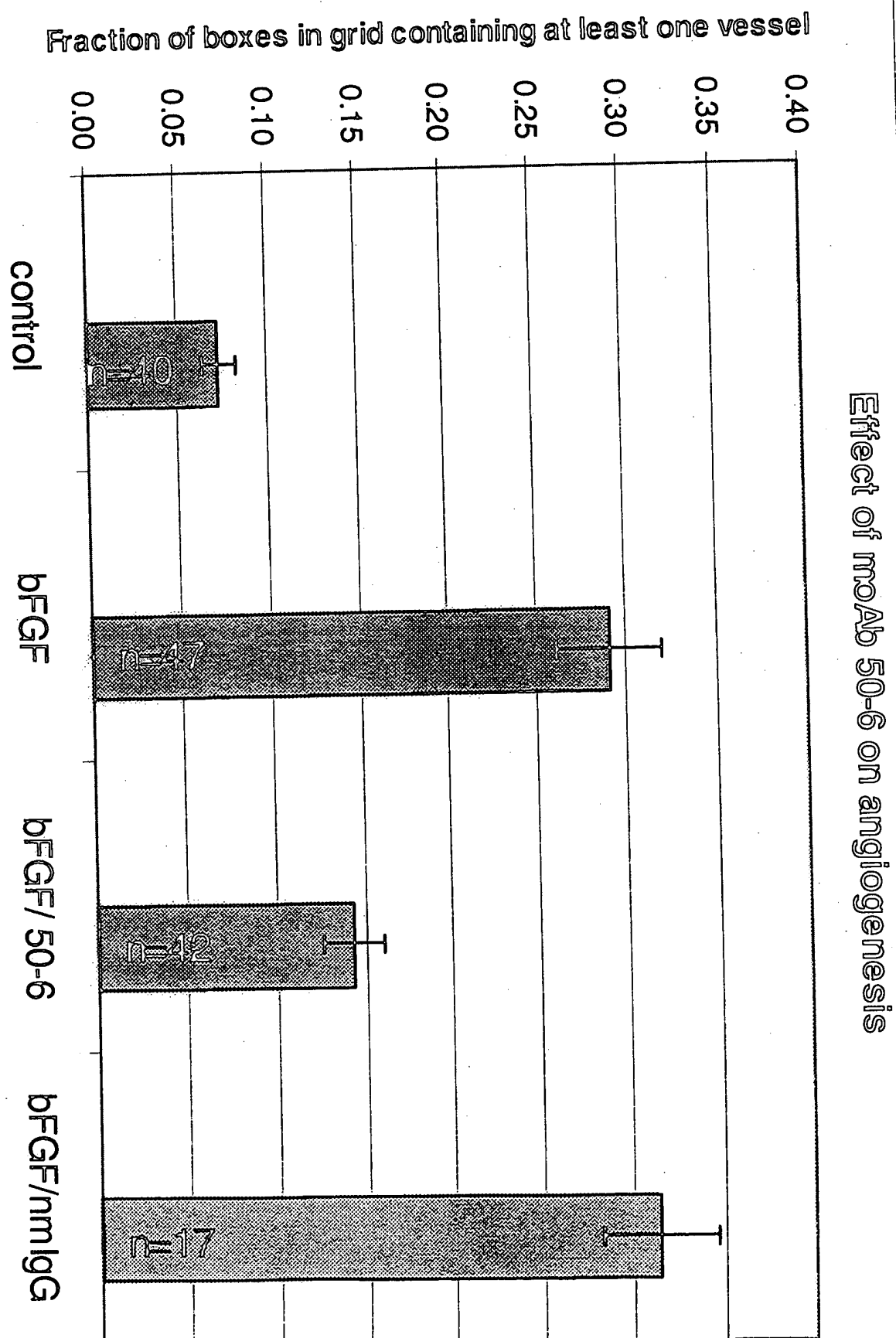


FIGURE 18

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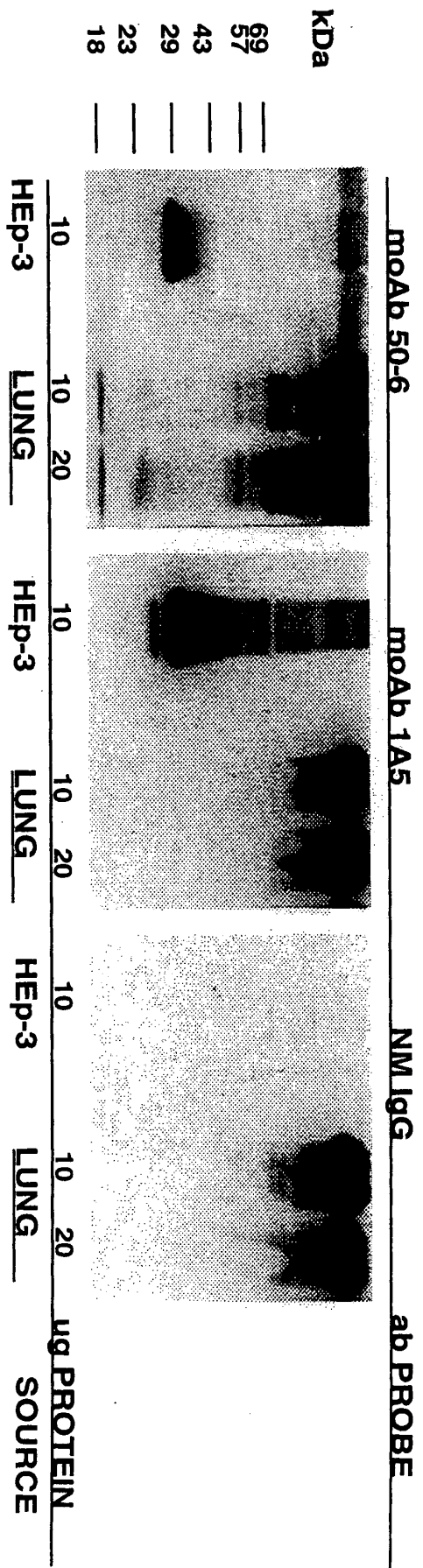


FIGURE 19

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human	(1)	MGEFNEKKTTCGTVCLKYLLFTYNCCFWLAGLAVMAVGIWTLALKSDYIS
mouse	(1)	MGEFNEKKaTCGTVCLKYLLFTYNCCFWLAGLAVMAVGIWTLALKSDYIS
human	(51)	LLASGTYLATAYILVVAGTVVMVTGVLGCCATFKERRNLLRLYFILLII
mouse	(51)	<u>LLASsTYLATAYILVVAGvVVMVTGVLGCCATFKERRNLLRLYFILLII</u>
human	(101)	FLLEIIAGILAYAYYQQLNTELKENLKDTMTKRYHQPGHEAVTSAVDQLO
mouse	(101)	<u>FLLEIIAGILAYvYYQQLNTELKENLKDTMvKRYHOSGHEqVsSAVDkLO</u>
human	(151)	QEFHCCGSNNSQDWRDSEWIRSQEAGGRVVPDSCCKTVVALCGQRDHASN
mouse	(151)	<u>QEFHCCGSNNSODWqDSEWIRSqEAdsRVVPDSCCKTmVAqCGkRDHASN</u>
human	(201)	IYKVEGGCITKLETFIQEHRLRVIGAVGIGIACVQVFGMIFTCCLYRSLKL
mouse	(201)	<u>IYKVEGGCITKLETFIQEHRLRVIGAVGIGIACVQVFGMIFTCCLYRSLKL</u>
human	(251)	EHY
mouse	(251)	EHY

FIGURE 20

PETA-3	(113)	AYYQQLNTELKENLKDTMTKRYHQPGHEAVTSAVDQLQQEFHCCGSNNSQ
CD9	(111)	SHKDEVIKEVQEFYKDTYNKLKT--KDEPQRETLKAIHYALNCCGLAGGV
CD63	(105)	VFRDKVMSEFNNNFRQQMENYP---KNNHTASILDRMQADFKCCGAANYT
PETA-3	(163)	DWRDSEWIRSQEAGGRVVPDSCCKTVVALCGQORDHASNIYKVEGGCITKL
CD9	(159)	EQF-----ISDICPKKDVLRTF-----TVKSCPDAI
CD63	(152)	DWEKIPSMKSNR-----VPDSCCINVTVGCG--INFNEKAIHKEGCVEKI
PETA-3	(213)	ETFIQEHLR
CD9	(185)	KEVFDNKFH
CD63	(196)	GGWLRKNVL

FIGURE 21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13480

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 5/12; C07K 16/30; A61K 39/395; C12N 15/11

US CL : 530/388.1, 388.8; 424/130.1; 536/23.1, 23.53; 435/326, 344

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/388.1, 388.8; 424/130.1; 536/23.1, 23.53; 435/326, 344

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FITTER et al. Molecular cloning of cDNA encoding a novel platelet-endothelial cell tetra-span antigen, PETA-3. Blood. 15 August 1995, Vol. 86, No. 4, pages 1348-1355, especially pages 1351-1352	2, 4, 6, 10, 15, 16, 17, 18
X ----- Y	YANEZ-MO et al. Regulation of endothelial cell motility by complexes of tetraspan molecules CD81/TPA1 and CD151/PETA-3 with $\alpha 3\beta 1$ integrin localized at endothelial lateral junctions. The Journal of Cell Biology. 04 May 1998, Vol. 141, No. 3, pages 791-804, especially abstract and pages 792 and 797.	2, 4, 6, 16, 26 ----- 10, 15, 17, 18, 20

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

27 AUGUST 1999

Date of mailing of the international search report

21 OCT 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer
Larry R. Helms

JOYCE BRIDGERS
PARALEGAL SPECIALIST
CHEMICAL MATRIX

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13480

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BROOKS et al. Subtractive immunization yields monoclonal antibodies that specifically inhibit metastasis. The Journal of Cell Biology. September 1993, Vol. 122, No. 6, pages 1351-1359, see especially Table IV and pages 1355.	16-21
A,P	SINCOCK et al. PETA-3/CD151, a member of the transmembrane 4 superfamily, is localised to the plasma membrane and endocytic system of endothelial cells, associates with multiple integrins and modulates cell function. Journal of Cell Science. 1999, Vol. 112, pages 833-844, see entire article.	2, 4, 6, 10, 15-21, 26

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/13480

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1, 3, 5, 9, 23, 24
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Please See Extra Sheet.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
2, 4, 6, 10, 15-21, 26

Remark on Protest

☐
☐

- The additional search fees were accompanied by the applicant's protest.
No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/13480

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

WPIDS, WEST, MEDLINE, CAPLUS, BIOSIS

search terms: PETA-3, antibody, CD151, anti-PETA-3 antibody, metastatic tumor, 1A-5, angiogenesis

BOX I. OBSERVATIONS WHERE CLAIMS WERE FOUND UNSEARCHABLE

2. Where no meaningful search could be carried out, specifically:

Claim 1 lacks the ATCC deposit number, antigen bound and structural and functional limitations necessary to properly search the claim. Dependent claims 3, 5, 9, 23 and 24 do not correct this deficiency. The disclosure provides a partial amino acid SEQ ID NO:3 (page 17), however, this structural feature of the antigen is not searchable because application is not in compliance with sequence requirements. The lab designation 41-2 is not sufficient to conduct a proper search on the claims. Claims 2, 4, 6, 19, 21 were searchable because the disclosure teaches that 50-6 binds to the PETA-3 antigen (page 16). Claim 15, 17, and 18 been searched to the extent that they read on the PETA-3 antigen.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

Group I, claim(s) 2, 4, 6, 10, 15-21 and 26, drawn to antibody, hybridoma, methods of detection and methods of treatment using such.

Group II, claim(s) 7-8, 11-14, drawn to antigen and nucleic acid encoding such.

Group III, claim(s) 22, 25, drawn to method of treating a metastatic tumor by administering an antisense molecule.

The inventions listed as Groups I, II and III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I recites the special technical features of a hybridoma cell line and a method of diagnosis that are not present in groups II or III. Group II recites the special technical feature of a host cell transformed with an expression vector containing nucleic acid encoding an antigen which is not present in Groups I or III. Group III recites the special technical feature of an antisense molecule that is not recited in Groups I or II. Thus Groups I, II and III do not relate to a single inventive concept under PCT Rule 13.1.

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